

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner
 US Department of Commerce
 United States Patent and Trademark
 Office, PCT
 2011 South Clark Place Room
 CP2/5C24
 Arlington, VA 22202
 ETATS-UNIS D'AMERIQUE
 in its capacity as elected Office

Date of mailing (day/month/year) 01 February 2001 (01.02.01)	
International application No. PCT/US99/05853	Applicant's or agent's file reference
International filing date (day/month/year) 12 March 1999 (12.03.99)	Priority date (day/month/year) 12 March 1998 (12.03.98)
Applicant PAPADOPOULOS, Vassilios et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
13 September 1999 (13.09.99)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Maria Victoria CORTIELLO Telephone No.: (41-22) 338.83.38
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INTERNATIONAL COOPERATION TREATY

PCT

NOTICE INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:

PRATT & ASSOCIATES, INC.
10821 Hillbrooke Lane
Potomac, MD 20854
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 16 September 1999 (16.09.99)		
Applicant's or agent's file reference		IMPORTANT NOTICE
International application No. PCT/US99/05853	International filing date (day/month/year) 12 March 1999 (12.03.99)	
Priority date (day/month/year) 12 March 1998 (12.03.98)		
Applicant GEORGETOWN UNIVERSITY MEDICAL CENTER et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
AU,EP,JP,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
CA

The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
16 September 1999 (16.09.99) under No. WO 99/46575

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

PCT COOPERATION TREATY

PCT

NOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

From the INTERNATIONAL BUREAU


To:

PRATT & ASSOCIATES, INC.
10821 Hillbrooke Lane
Potomac, MD 20854
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 17 May 1999 (17.05.99)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference	
International application No. PCT/US99/05853	International filing date (day/month/year) 12 March 1999 (12.03.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 12 March 1998 (12.03.98)
Applicant GEORGETOWN UNIVERSITY MEDICAL CENTER et al	

- The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
- This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
- An asterisk (*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
- The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
12 Marc 1998 (12.03.98)	60/077,753	US	27 Apr 1999 (27.04.99)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 740.14.35	Authorized officer Marc Salzman  Telephone No. (41-22) 338.83.38
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TENT COOPERATION TREA

PCT

NOTIFICATION OF RECEIPT OF
RECORD COPY

(PCT Rule 24.2(a))

From the INTERNATIONAL BUREAU

To:

PRATT & ASSOCIATES, INC.
10821 Hillbrooke Lane
Potomac, MD 20854
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 11 May 1999 (11.05.99)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference	International application No. PCT/US99/05853

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

GEORGETOWN UNIVERSITY MEDICAL CENTER (for all designated States except US)
PAPADOPOULOS, Vassilios et al (for US)

International filing date : 12 March 1999 (12.03.99)
Priority date(s) claimed : 12 March 1998 (12.03.98)
Date of receipt of the record copy
by the International Bureau : 23 April 1999 (23.04.99)
List of designated Offices :

EP : AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE
National : AU, CA, JP, US

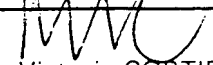
ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

- ☒ time limits for entry into the national phase
☒ confirmation of precautionary designations
☒ requirements regarding priority documents

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 740.14.35	Authorized officer:  Maria Victoria CORTIELLO Telephone No. (41-22) 338.83.38
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INFORMATION ON TIME LIMITS FOR ENTERING THE NATIONAL PHASE

The applicant is reminded that the "national phase" must be entered before each of the designated Offices indicated in the Notification of Receipt of Record Copy (Form PCT/IB/301) by paying national fees and furnishing translations, as prescribed by the applicable national laws.

The time limit for performing these procedural acts is **20 MONTHS** from the priority date or, for those designated States which the applicant elects in a demand for international preliminary examination or in a later election, **30 MONTHS** from the priority date, provided that the election is made before the expiration of 19 months from the priority date. Some designated (or elected) Offices have fixed time limits which expire even later than 20 or 30 months from the priority date. In other Offices an extension of time or grace period, in some cases upon payment of an additional fee, is available.

In addition to these procedural acts, the applicant may also have to comply with other special requirements applicable in certain Offices. **It is the applicant's responsibility** to ensure that the necessary steps to enter the national phase are taken in a timely fashion. Most designated Offices do not issue reminders to applicants in connection with the entry into the national phase.

For detailed information about the procedural acts to be performed to enter the national phase before each designated Office, the applicable time limits and possible extensions of time or grace periods, and any other requirements, see the relevant Chapters of Volume II of the PCT Applicant's Guide. Information about the requirements for filing a demand for international preliminary examination is set out in Chapter IX of Volume I of the PCT Applicant's Guide.

GR and ES became bound by PCT Chapter II on 7 September 1996 and 6 September 1997, respectively, and may, therefore, be elected in a demand or a later election filed on or after 7 September 1996 and 6 September 1997, respectively, regardless of the filing date of the international application. (See second paragraph above.)

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

CONFIRMATION OF PRECAUTIONARY DESIGNATIONS

This notification lists only specific designations made under Rule 4.9(a) in the request. It is important to check that these designations are correct. Errors in designations can be corrected where precautionary designations have been made under Rule 4.9(b). The applicant is hereby reminded that any precautionary designations may be confirmed according to Rule 4.9(c) before the expiration of 15 months from the priority date. If it is not confirmed, it will automatically be regarded as withdrawn by the applicant. There will be no reminder and no invitation. Confirmation of a designation consists of the filing of a notice specifying the designated State concerned (with an indication of the kind of protection or treatment desired) and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.

REQUIREMENTS REGARDING PRIORITY DOCUMENTS

For applicants who have not yet complied with the requirements regarding priority documents, the following is recalled.

Where the priority of an earlier national, regional or international application is claimed, the applicant must submit a copy of the said earlier application, certified by the authority with which it was filed ("the priority document") to the receiving Office (which will transmit it to the International Bureau) or directly to the International Bureau, before the expiration of 16 months from the priority date, provided that any such priority document may still be submitted to the International Bureau before that date of international publication of the international application, in which case that document will be considered to have been received by the International Bureau on the last day of the 16-month time limit (Rule 17.1(a)).

Where the priority document is issued by the receiving Office, the applicant may, instead of submitting the priority document, request the receiving Office to prepare and transmit the priority document to the International Bureau. Such request must be made before the expiration of the 16-month time limit and may be subjected by the receiving Office to the payment of a fee (Rule 17.1(b)).

If the priority document concerned is not submitted to the International Bureau or if the request to the receiving Office to prepare and transmit the priority document has not been made (and the corresponding fee, if any, paid) within the applicable time limit indicated under the preceding paragraphs, any designated State may disregard the priority claim, provided that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity to furnish the priority document within a time limit which is reasonable under the circumstances.

Where several priorities are claimed, the priority date to be considered for the purposes of computing the 16-month time limit is the filing date of the earliest application whose priority is claimed.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No.	International filing date (day/month/year)	(Earliest) Priority Date (day/month/year)
PCT/US 99/ 05853	12/03/1999	12/03/1998
Applicant		
GEORGETOWN UNIVERSITY MEDICAL CENTER		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 7 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☒ furnished subsequently to this Authority in written form.

☒ furnished subsequently to this Authority in computer readable form.

☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☐ the text is approved as submitted by the applicant.

☒ the text has been established by this Authority to read as follows:

PEPTIDES CONTAINING A COLESTEROL RECOGNITION SEQUENCE AND THEIR USES

5. With regard to the **abstract**,

☐ the text is approved as submitted by the applicant.

☒ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.



FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 23-24, 29 (at least partially), 32-33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 1-36

The claims of the present application were searched only insofar as further defined for the following reasons.

Claims 1-8 refer to a "consensus sequence". A consensus sequence is considered to be a descriptor of a substance which should be defined by physico-chemical and functional parameters. As such a consensus sequence has no meaning and has been considered to fall under the category "presentation of information" and is thus excluded from search according to Rule 39(V) PCT. Compounds including a consensus sequence and their uses etc. are instead searchable if their definition respects the requirements of Art. 6 and Rule 6 PCT.

Claims 9-26 were searched only insofar described by the real examples present in the text. The general claims 9 and 20 are so broad that a lack of clarity can be immediately recognized, contravening the requirements of Art. 6 and Rule 6 PCT.

Claims 27-28, described only by a functional feature which is in itself a desideratum, were not searched as contravening Art. 6 and Rule 6 PCT to an intolerable extent.

Claims 29-36 were searched completely. Due to the presence of two claims, the claims from 31(2) through 36 have been renumbered 32-37.



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 05853

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

Peptides containing a cholesterol interaction/recognition consensus sequence is described and methods of use thereof.



09/623 922

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

500

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 September 1999 (16.09.1999)

PCT

(10) International Publication Number
WO 99/46575 A3

(51) International Patent Classification⁶: C07K 14/705,
C12N 15/82, G01N 33/68, C12N 15/11

Vassilios [US/US]; 15417 Peach Leaf Drive, North Potomac, MD 20878 (US). LI, Hua [CN/US]; Apartment 102, 1319 Fort Myer Drive, Arlington, VA 22209 (US).

(21) International Application Number: PCT/US99/05853

(74) Agent: PRATT & ASSOCIATES, INC.; 10821 Hillbrooke Lane, Potomac, MD 20854 (US).

(22) International Filing Date: 12 March 1999 (12.03.1999)

(25) Filing Language: English

(81) Designated States (*national*): AU, CA, JP, US.

(26) Publication Language: English

(84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

(30) Priority Data:
60/077,753 12 March 1998 (12.03.1998) US

Published:
— With international search report.

(71) Applicant (*for all designated States except US*):
GEORGETOWN UNIVERSITY MEDICAL CENTER
[US/US]; Building D, Suite 177, 4000 Reservoir Road,
N.W., Washington, DC 20007 (US).

(88) Date of publication of the international search report:
28 June 2001

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): PAPADOPOULOS,

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PEPTIDES CONTAINING A CHOLESTEROL RECOGNITION SEQUENCE AND THEIR USES

(57) Abstract: Peptides containing a cholesterol interaction/recognition consensus sequence is described and methods of use thereof.

WO 99/46575 A3

INTERNATIONAL SEARCH REPORT

Intern. Application No.

PCT/US 99/05853

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/705 C12N15/82 G01N33/68 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J R GLENNEY JR. : "The sequence of human caveolin reveals identity with VIP21, a component of transport vesicles" FEBS LETTERS., vol. 314, no. 1, 1992, pages 45-48, XP002154826 ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM., NL ISSN: 0014-5793 the whole document --- -/--	9-20

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

11 December 2000

Date of mailing of the international search report

02/01/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Masturzo, P



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/05853

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>T P BOYLE & K R MAROTTI: "Structure of the murine gene encoding apolipoprotein A-1" GENE., vol. 117, 1992, pages 243-247, XP002154827 ELSEVIER BIOMEDICAL PRESS. AMSTERDAM., NL ISSN: 0378-1119 cited in the application the whole document</p> <p style="text-align: center;">---</p>	9-20
X	<p>V PAPADOPOULOS ET AL.: "Targeted disruption of the peripheral-type benzodiazepine receptor gene inhibits steroidogenesis in the R2C Lxdig tumor cell line" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 22, no. 51, 19 December 1997 (1997-12-19), pages 32129-22135, XP002114579 AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD., US ISSN: 0021-9258 the whole document</p> <p style="text-align: center;">---</p>	31
A	<p>DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; PIKULEVA I A ET AL: "Active-site topology of bovine cholesterol side-chain cleavage cytochrome P450 (P450scc) and evidence for interaction of tyrosine 94 with the side chain of cholesterol." retrieved from STN Database accession no. 96004795 XP002154829 & ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, (1995 SEP 10) 322 (1) 189-97. , abstract</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	29, 30

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INTERNATIONAL SEARCH REPORT

Interr. nal Application No

PCT/US 99/05853

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; SU P ET AL: "A cDNA encoding a rat mitochondrial cytochrome P450 catalyzing both the 26-hydroxylation of cholesterol and 25-hydroxylation of vitamin D3: gonadotropic regulation of the cognate mRNA in ovaries." retrieved from STN Database accession no. 91083838 XP002154830 & DNA AND CELL BIOLOGY, (1990 NOV) 9 (9) 657-67. , abstract</p>	9-20
X	<p>DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; FARGES, ROSELI ET AL: "Site-directed mutagenesis of the peripheral benzodiazepine receptor: identification of amino acids implicated in the binding site of Ro5-4864" retrieved from STN Database accession no. 122:122952 CA XP002154831 & MOL. PHARMACOL. (1994), 46(6), 1160-7 , 1994, abstract</p>	31
P,X	<p>H LI & V PAPADOPOULOS: "Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern" ENDOCRINOLOGY., vol. 139, no. 12, 1999, pages 4991-4997, XP002154828 BALTIMORE, MD., US ISSN: 0013-7227 the whole document</p>	9-36

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/05853

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>M HARDWICK ET AL.: "Peripheral-type benzodiazepine receptor (PBR) in human breast cancer: correlation of breast cancer cell aggressive phenotype with PBR expression, nuclear localization, and PBR-mediated cell proliferation and nuclear transport of cholesterol "</p> <p>CANCER RESEARCH., vol. 59, 15 February 1999 (1999-02-15), pages 831-842, XP002114583 AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD., US ISSN: 0008-5472 the whole document</p> <p style="text-align: center;">---</p>	31
X	<p>V PAPADOPOULOS : "Structure and function of the peripheral-type benzodiazepine receptor in steroidogenic cells"</p> <p>PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY & MEDICINE., vol. 217, no. 2, February 1998 (1998-02), pages 130-142, XP002154897 ACADEMIC PRESS INC. NEW YORK., US ISSN: 0037-9727 the whole document</p> <p style="text-align: center;">---</p>	31
X	<p>K E KRUEGER & V PAPADOPOULOS: "Peripheral-type benzodiazepine receptors mediate translocation from outer to inner mitochondrial membranes in adrenocortical cells"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 65, no. 25, 5 September 1990 (1990-09-05), pages 15015-15022, XP002133901 AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD., US ISSN: 0021-9258 the whole document</p> <p style="text-align: center;">---</p>	9-20
A	<p>H MIETTINEN ET AL.: "Expression of peripheral-type benzodiazepine receptor and diazepam binding inhibitor in human astrocytomas; relationship to cell proliferation"</p> <p>CANCER RESEARCH., vol. 55, 15 June 1995 (1995-06-15), pages 2691-2695, XP002114581 AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD., US ISSN: 0008-5472 the whole document</p> <p style="text-align: center;">-----</p>	9-36

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 23-24, 29 (at least partially), 32-33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 1-36

The claims of the present application were searched only insofar as further defined for the following reasons.

Claims 1-8 refer to a "consensus sequence". A consensus sequence is considered to be a descriptor of a substance which should be defined by physico-chemical and functional parameters. As such a consensus sequence has no meaning and has been considered to fall under the category "presentation of information" and is thus excluded from search according to Rule 39(V) PCT. Compounds including a consensus sequence and their uses etc. are instead searchable if their definition respects the requirements of Art. 6 and Rule 6 PCT.

Claims 9-26 were searched only insofar described by the real examples present in the text. The general claims 9 and 20 are so broad that a lack of clarity can be immediately recognized, contravening the requirements of Art. 6 and Rule 6 PCT.

Claims 27-28, described only by a functional feature which is in itself a desideratum, were not searched as contravening Art. 6 and Rule 6 PCT to an intolerable extent.

Claims 29-36 were searched completely. Due to the presence of two claims, the claims from 31(2) through 36 have been renumbered 32-37.



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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 50 APR 2003

09623922

Applicant's or agent's file reference 23521-0118	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/05853	International filing date (day/month/year) 12 March 1999 (12.03.1999)	Priority date (day/month/year) 12 March 1998 (12.03.1998)
International Patent Classification (IPC) or national classification and IPC IPC(7): C07K 14/705; G01N 33/68; C12N 15/11, 15/82 and US Cl.: 536 23.1; 530 350; 435 7.1, 468; 436 500;		
Applicant GEORGETOWN UNIVERSITY MEDICAL CENTER		
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of <u>6</u> sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of <u>—</u> sheets.</p>		
<p>3. This report contains indications relating to the following items:</p> <p>I <input checked="" type="checkbox"/> Basis of the report</p> <p>II <input type="checkbox"/> Priority</p> <p>III <input checked="" type="checkbox"/> Non-establishment of report with regard to novelty, inventive step and industrial applicability</p> <p>IV <input type="checkbox"/> Lack of unity of invention</p> <p>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p>VI <input type="checkbox"/> Certain documents cited</p> <p>VII <input checked="" type="checkbox"/> Certain defects in the international application</p> <p>VIII <input checked="" type="checkbox"/> Certain observations on the international application</p>		
Date of submission of the demand 13 SEPTEMBER 1999	Date of completion of this report 17 April 2003 (17.04.2003)	
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Christopher Nichols, Ph.D. Telephone No. 703-305-3955	

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/05853

I. Basis of the report

1. With regard to the elements of the international application:*

- ☒ the international application as originally filed.
- ☒ the description:
pages 1-52 as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the claims:
pages 53-58, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☒ the drawings:
pages 1-8, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____
- ☐ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in printed form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

- ☐ the description, pages NONE
- ☐ the claims, Nos. NONE
- ☐ the drawings, sheets/fig NONE

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/05853

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The question whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application,

☒ claims Nos. 1-8

because:

☐ the said international application, or the said claim Nos. _____ relate to the following subject matter which does not require international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. _____ are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 1-8

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/05853

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims <u>9-36</u>	YES
	Claims <u>NONE</u>	NO
Inventive Step (IS)	Claims <u>9-36</u>	YES
	Claims <u>NONE</u>	NO
Industrial Applicability (IA)	Claims <u>9-36</u>	YES
	Claims <u>NONE</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 9-36 the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest the claimed nucleic acid, host cells, peptide, molecules, or transgenic plants.
Claims 9-36 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

----- NEW CITATIONS -----

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/05853

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/05853

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claims 1-8 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims are indefinite for the following reason(s): the claims refer to a "consensus sequence", the metes and bounds of which are not defined.

Claims 9-26 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims are indefinite for the following reason(s): claims 9 and 20 are so broad as to lack clarity.

Claims 27-28 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims are indefinite for the following reason(s): claims 27 and 28 do not adequately describe the invention.

PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US99/05853 (22) International Filing Date: 12 March 1999 (12.03.99) (30) Priority Data: 60/077,753 12 March 1998 (12.03.98) US (71) Applicant (for all designated States except US): GEORGETOWN UNIVERSITY MEDICAL CENTER [US/US]; Building D, Suite 177, 4000 Reservoir Road, N.W., Washington, DC 20007 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): PAPADOPOULOS, Vasilios [US/US]; 15417 Peach Leaf Drive, North Potomac, MD 20878 (US). LI, Hua [CN/US]; Apartment 102, 1319 Fort Myer Drive, Arlington, VA 22209 (US). (74) Agent: PRATT & ASSOCIATES, INC.; 10821 Hillbrooke Lane, Potomac, MD 20854 (US).		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: CHOLESTEROL RECOGNITION SEQUENCE (57) Abstract A cholesterol interaction/recognition consensus sequence is described and methods of use thereof.		

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TITLE OF THE INVENTION**Cholesterol Recognition Sequence**

5

INTRODUCTION

10 The primary point of control in the acute
stimulation of steroidogenesis by hormones involves
the first step in this biosynthetic pathway where
cholesterol is converted to pregnenolone by the C₂₇
cholesterol side chain cleavage cytochrome P-450
enzyme (P-450scc) and auxiliary electron transferring
15 proteins, localized on inner mitochondrial membranes
(IMM) (Simpson, E.R. and Waterman, M. R., 1983, *Can.*
J. Biochem. Cell. Biol. 61:692-717; Jefcoate, C. R. et
al. 1992, *J. Steroid Biochem. Molec. Biol.* 43: 751-
767). More detailed studies have shown that the
20 reaction catalyzed by P-450scc is not the rate-
limiting step in the synthesis of steroid hormones.
Rather, the rate-limiting step is the transport of the
precursor, cholesterol, from intracellular sources to
the IMM. This hormone-dependent transport mechanism
25 was shown to be localized in the mitochondrion
(Simpson and Waterman, 1983, *supra*; Jefcoate et al.,
1992, *supra*). All documents cited herein *supra* and
infra are hereby incorporated in their entirety by
reference thereto.

30 The peripheral-type benzodiazepine receptor (PBR)
was originally discovered because it binds the
benzodiazepine diazepam with relatively high affinity
(Papadopoulos, V. 1993, *Endocr. Rev.* 14:222-240).
Benzodiazepines are among the most highly prescribed

drugs due to their pharmacological actions in relieving anxiety mediated through modulating the activity of γ -aminobutyric acid receptors in the central nervous system (Costa, E. and Guidotti, A. 1979, *Annu. Rev. Pharmacol. Toxicol.* 19:531-545). PBR is another class of binding sites for benzodiazepines distinct from the aforementioned neurotransmitter receptors. Further studies demonstrated that in addition to benzodiazepines, PBR binds other classes of organic compounds with high affinity (Papadopoulos, 1993, *supra*). PBR, although present in all tissues examined, was found to be particularly high in steroid producing tissues, where it was primarily localized in the outer mitochondrial membrane (OMM) (Anholt, R.R.H. et al. 1986, *J. Biol. Chem.* 261:576-583). An 18 kDa isoquinoline-binding protein was identified as PBR, cloned and expressed (Papadopoulos, V. 1998, *Proc Soc. Exp. Biol. Med.* 217:130-142). It was then demonstrated that PBR is a functional component of the steroidogenic machinery (Papadopoulos, 1998, *supra*; Papadopoulos V. et al. 1990, *J. Biol. Chem.* 265:3772-3779) mediating cholesterol delivery from the outer to the inner mitochondrial membrane (Krueger, K. E. and Papadopoulos, V. 1990, *J. Biol. Chem.* 265:15015-15022). Further studies demonstrated that pharmacologically induced reduction of adrenal PBR levels *in vivo* resulted in decreased circulating glucocorticoid levels (Papadopoulos, V. 1998, *supra*). In addition, targeted disruption of the PBR gene in Leydig cells resulted in the arrest of cholesterol transport into mitochondria and steroid formation; transfection of the mutant cells with a PBR cDNA rescued steroidogenesis (Papadopoulos, V. et al. 1997, *J. Biol. Chem.* 272:32129-32135). How PBR affected

cholesterol transport and whether or not PBR directly interacted with cholesterol was not known.

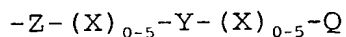
SUMMARY OF THE INVENTION

5 Based on the known amino acid sequence of the human and mouse 18 kDa PBR protein, a three dimensional model of this receptor protein was developed using molecular dynamics simulations (Bernassau, J. M. *et al.* 1993, *J. Mol. Graph.* 11:236-
10 245; Papadopoulos, V. 1996, In: Payne A. H., Hardy, M. P., Russell, L. D. (eds) The Leydig Cell. Cashe River Press, Vienna, IL, pp 598-628). Analysis of the three dimensional structure indicated that both the human and mouse PBR could possibly accomodate cholesterol
15 and function as a channel. In this invention is described the testing and proof of this hypothesis.

 In the present invention, we identify a cholesterol recognition amino acid sequence on PBR, common to all proteins shown to interact with
20 cholesterol, and demonstrate that PBR functions as a cholesterol channel-like structure, mediating cholesterol transport across membranes.

 Cholesterol, coming from various intracellular sources, is recognized by the cholesterol
25 recognition/interaction amino acid consensus pattern present in the carboxy-terminal of PBR in the outer mitochondrial membrane (OMM). This pool of cholesterol enters in the (OMM) at the PBR sites where it remains without mixing with other membrane
30 components. Ligand binding to the receptor induces the release of this cholesterol. When it is released, the cholesterol can be accessed by the P450scc and cleaved into pregnenolone, precursor of all steroids.

Therefore, it is an object of the present invention to provide a consensus sequence for the recognition/interaction of cholesterol comprising



5 wherein Z represents a neutral and hydrophobic amino acid, such as Leucine or Valine, Y represents a neutral and polar amino acid, such as Tyrosine, Q represents a basic amino acid, such as Arginine or Lysine and X represents any amino acid. The presence
10 of the consensus sequence in a protein infers the likelihood of interaction with cholesterol.

It is another object of the present invention to provide a nucleotide sequence encoding the consensus sequence described above, and vectors incorporating
15 all or part of said sequence, and cells, prokaryotic and eukaryotic, transformed or transfected with said vectors, for use in the production of peptides having a cholesterol interaction/recognition sequence. The transformed or transfected cells are useful for
20 screening agents or drugs which alter cholesterol interaction/recognition, and uptake of cholesterol.

It is yet another object of the present invention to provide a method for identifying whether or not a protein recognizes or interacts with cholesterol said
25 method comprising identifying the presence or absence of the above-mentioned cholesterol recognition/interaction consensus sequence wherein the presence of the sequence is an indication of the likelihood that the protein recognizes/interacts with cholesterol.

30 It is yet another object of the present invention to provide a method for conferring to a molecule the ability to interact with cholesterol by introducing into a molecule or polypeptide, natural or synthetic, a cholesterol recognition/interaction sequence such

that the protein or polypeptide, natural or synthetic, is now able to recognize/interact with cholesterol.

It is still another object of the present invention to provide a molecule, natural or synthetic
5 comprising the cholesterol recognition/interaction sequence described above.

It is a further object of the present invention to provide a molecule which blocks or competes with the cholesterol interaction/recognition ability of the
10 cholesterol recognition/interaction consensus described above. The molecule can be an antibody, a peptide, a peptide comprising the cholesterol interaction/recognition consensus sequence, or drug.

It is yet another object of the present invention
15 to provide a method for altering the cholesterol binding ability of molecules which contain the cholesterol recognition/interaction consensus described above, comprising altering said site such that cholesterol recognition is reduced, eliminated or
20 increased. Cholesterol recognition can be increased by providing a perfect cholesterol pocket, or alternatively, by providing more than one consensus sequence in a protein or molecule.

It is further another object of the present
25 invention to provide a PBR with reduced ability to recognize and interact with cholesterol by modifying the cholesterol recognition/interaction site of PBR, for example by changing amino acid 153 from tyrosine to serine, or by changing amino acid 155 from arginine
30 to leucine. These modification or similar modifications may be used for reducing the cholesterol recognition/interaction or binding of other proteins or agents which contain the cholesterol interaction/recognition consensus sequence described
35 above.

It is another object of the present invention to provide a method for increasing the presence of cholesterol inside the cell by introducing a PBR ligand such that the cholesterol is released from PBR.

5 It is another object of the present invention to provide a method for decreasing the presence of cholesterol inside the cell by introducing an agent which inhibits PBR ligand binding to PBR thereby inhibiting the release of cholesterol from PBR. Said
10 agent can be any molecule, peptide, or drug.

It is yet another object of the present invention to provide a method for increasing the presence of cholesterol inside the cell by introducing an agent which results in overexpression of the PBR, or
15 increase in a peptide comprising the cholesterol interaction/recognition sequence, by gene therapy for example, or altering the distribution and compartmentalization of cholesterol by targetting the cholesterol interaction/recognition sequence to a
20 specific organelle in the cell, i.e. by addign a signal sequence specific for nuclear insertion at the N-terminus

It is yet another object of the present invention to provide a method for increasing pregnenolone
25 production in a steroidogenic cell, comprising providing a PBR ligand to said cell such that PBR releases cholesterol which is then available for cleavage into pregnenolone. Alternatively, a peptide comprising the cholesterol interaction/recognition
30 sequence can be provided to a steroidogenic cell, i.e. by gene therapy, thereby increasing the presence of cholesterol in a steroidogenic cell, resulting in an increase of pregnenolone production.

It is another object of the present invention to
35 provide a method for decreasing pregnenolone

production in a steroidogenic cell, comprising providing a PBR ligand inhibitor to said cell such that release of PBR bound cholesterol is inhibited and therefore cleavage of the cholesterol to pregnenolone is inhibited. Alternatively, a peptide comprising the cholesterol interaction/recognition sequence can be used to compete the cholesterol from its binding site on the endogenous PBR receptor. This method is useful for reducing disease symptoms resulting from increased production of steroids, for example, stress and Cushing's disease.

It is still another object of the present invention to provide a method for increasing steroid production in a steroidogenic cell, comprising providing a PBR ligand to said cell such that PBR releases cholesterol which is then available for cleavage into pregnenolone and used as the precursor of all steroids.

It is another object of the present invention to provide a method for reducing steroid production in a steroidogenic cell, comprising providing an agent which inhibits PBR ligand binding to PBR such that PBR-bound cholesterol is not released and said cholesterol is not cleaved into pregnenolone, the precursor of all steroids.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1 shows effect of PBR ligands on MA-10 Leydig cell cholesterol transport and pregnenolone formation. Mitochondria from MA-10 Leydig cells were incubated with ³H-cholesterol in the presence or

absence of the indicated compounds. ^3H -Pregnenolone formed was measured as described in Materials and Methods. Data (means \pm SD) shown are representative of two to four independent experiments, each having
5 triplicate assays. The effect seen was statistically significant at all times ($P < 0.001$).

Figure 2 A, B, and C show the expression of recombinant PBR in bacteria. Recombinant mPBR
10 expression vector was developed and used to transform the BL21(DE3) *Escherichia coli* strain as described in Materials and Methods. The expression of recombinant mPBR protein was induced by 1 mM IPTG.

A) PBR protein expression was monitored by SDS-
15 PAGE followed by Coomassie Blue staining or immunoblot analysis. 1, Control; 2 and 3, two different preparations of IPTG-treated bacteria.

B) Binding specificity of the IPTG-induced PBR in *Escherichia coli*. Specific binding of [^3H]PK11195
20 (1.0 nM) was measured in the presence of the indicated concentrations of various ligands.

C) Scatchard representation of the specific binding of ^3H -PK11195 to IPTG-induced bacteria.

25 Figure 3. Characteristics of ^3H -cholesterol uptake by *E. coli* cells.

A) ^3H -Cholesterol uptake by *E. coli* cells was examined using increasing concentrations of control or IPTG-treated transformed bacteria incubated in the
30 presence of 6.7 nM ^3H -cholesterol (50.0 Ci/mmol) for 60 min. at 37 C. Specific cholesterol uptake is defined as IPTG-induce minus basal values.

B) ^3H -Cholesterol specific uptake examined at the indicated temperatures using 100 ug bacterial protein.

- C) Effect of energy poisons on ^3H -cholesterol uptake by IPTG-induced transformed bacteria.
- D) PBR expression induces uptake of cholesterol only. Bacteria were incubated under the same conditions as described above in the presence of the indicated radiolabeled steroid.
- E) ^3H -cholesterol specific uptake determined in the presence of increasing concentrations of ^3H -cholesterol.
- F) Ligand-induced release of cholesterol uptaken by the bacterial membranes. ^3H -cholesterol-labeled membranes of IPTG-induced transformed bacteria were incubated with increased concentrations of PK11195 or clonazepam, and the ^3H -cholesterol released was quantified. The results shown represent means \pm SD from an experiment performed in triplicate. Similar results were obtained in three other independent experiments.

Figure 4. Deletion mutation analysis of PBR function-PK11195 ligand binding and ^3H -cholesterol uptake by bacteria transfected with the wild-type and mutated PBRs. Left, The five transmembrane regions of PBR are shown as well as the location of each deletion undertaken. Right, The effect of each deletion on PK11195 ligand binding and cholesterol uptake is shown. Results shown are the means of triplicates. 100% of PK 11195 ligand binding corresponds to 280 ± 22 fmol/mg protein. 100% of specific cholesterol uptake corresponds to 1.35 ± 0.15 pmol/mg of protein.

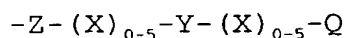
Figure 5. Identification of specific amino acids responsible for the uptake of cholesterol. ^3H -cholesterol uptake by bacteria transfected with the wild-type and point-mutated PBRs (bottom). The



expression of the proteins was induced by IPTG. 100% of specific cholesterol uptake corresponds to 1.2 ± 0.1 pmol of cholesterol per mg of protein. Immunoblot analysis of the mutated PBRs expressed by the bacteria examined for ^3H -cholesterol uptake, (top). Results shown are the means of triplicates.

DETAILED DESCRIPTION

In one embodiment, the present invention relates to a minimum amino acid sequence specific for recognition/interaction with cholesterol, namely the amino acid sequence



wherein Z represents a neutral and hydrophobic amino acid, such as Leucine, Valine, Alanine, Isoleucine, Methionine, Phenylalanine and Tryptophan, Y represents a neutral and polar amino acid, such as Tyrosine, Threonine, Serine, Glycine, Glutamine, Cysteine, Asparagine, Q represents a basic amino acid, such as Arginine, Lysine, or arginine, and X represents any amino acids selected from the group consisting of Alanine (Ala, A), Arginine (Arg, R), Asparagine (Asn, N), Aspartic acid (Asp, D), Cystein (Cys, C), Glutamine (Gln, Q), Glutamic acid (Glu, E), Glycine (Gly, G), Histidine (His, H), Isoleucine (Ile, I), Leucine (Leu, L), Lysine (Lys, K), Methionine (Met, M), Phenylalanine (Phe, F), Proline (Pro, P), Serine (Ser, S), Threonine (Thr, T), Tryptophan (Trp, W), Tyrosine (Tyr, Y), and Valine (Val, V).

For example, some of the sequences of the present invention include:

Leu Asn Tyr Tyr Val Trp Arg (SEQ ID NO:1)

Leu Asn Tyr Cys Val Trp Arg (SEQ ID NO:2)

Leu Asn Tyr Arg (SEQ ID NO:3)

Val Ala Tyr His Gln Tyr **Tyr** Gln **Arg** (SEQ ID
NO:4)

The number of X amino acids can be from none
5 (zero) to five, preferably 1-5, more preferably 2-5,
and most preferably 3-5 amino acids. These amino
acids function to provide proper folding of this
consensus sequence to produce the recognition/
interaction site. All peptides herein are written
10 NH₂...COOH and the amino acids are the naturally
occurring L isomers.

Without being held to a particular theory,
studies suggest that Leucine or Valine in position Z
will interact with the hydrophobic side chain of
15 cholesterol and Tyrosine at position Y will interact
with the polar 3'OH-group of cholesterol, whereas the
Arginine or Lysine at position Q may help create a
pocket.

This cholesterol recognition/interaction amino
20 acid sequence consensus pattern was found in molecules
shown or suggested to interact with cholesterol, such
as apolipoprotein A-1 (Boyle, T. P. and Marotti, K.R.,
1992, *Gene* 117, 243-247), caveolin (Murata, M. et al.
1995, *Proc. Natl. Aca. Sci. USA* 92, 10339-10343), DBI
25 (Papadopoulos, V. 1993, *Endocr. Rev.* 14, 222-240;
Papadopoulos, V. 1998, *Proc. Soc. Exp. Biol. Med.* 217,
130-142), steroidogenesis acute regulatory protein
(StAR) (Stocco, D. M. and Clark, B. J. 1996, *Endocr.*
Rev. 17, 221-244), hedgehog protein (Porter, J. A. et
30 al. 1996, *Science* 274, 255-259), cytochrome P450
C26/25 (Su, P. et al. 1990, *DNA Cell Biol.* 9-657-667),
annexin II (Harder, T. et al. 1997, *Mol. Biol. Cell* 8,
533-545), sterol carrier protein-2 (Colles, S. M. et
al. 1995, *Lipids* 30, 795-803), cholesterol 7 α -
35 monooxygenase (Kai, M. et al. 1995, *Lipid Res.* 36,

367-374), cholesterol oxidase (Ishizaki, T. et al. 1991, *J. Bacteriol.* 171, 596-601), cholesterol dehydrogenase (Horinouchi, S. et al. 1991, *Appl. Environ. Microbiol.* 57, 1386-1393), bile-salt-activated lipase precursor (cholesterol esterase) (Nilsson, J. et al. 1990, *Eur. J. Biochem.* 192, 543-550), and acyl-CoA cholesterol acyltransferase (Pape, M.E. et al. 1995, *J. Lipid Res.* 36, 823-838) as shown in Table 1.

10

Table 1: Identification of the cholesterol recognition/interaction amino acid consensus pattern.

	Mouse PBR	149- VLNYVWR (SEQ ID NO:5)
	Rat PBR	150- LNYVWR (SEQ ID NO:6)
15	Human PBR	150- LNYCVWR (SEQ ID NO:7)
	Bovine PBR	149- LNYR (SEQ ID NO:8)
	Rat P450scc	88-VAYHQYYQR (SEQ ID NO:9)
	Human P450scc	88-VAYHQYYQR (SEQ ID NO:10)
	Pig P450scc	88-VAYHZHYQK (SEQ ID NO:11)
20		
	Mouse apolipoprotein A-I	210- LNEYHTR (SEQ ID NO:12)
	Mouse caveolin	94- VTKYWFYR (SEQ ID NO:13)
	Human hedgehog	251- VFYVIETR (SEQ ID NO:14)
	Mouse DBI	25- LFIYSHFK (SEQ ID NO:15)
25	Mouse StAR	6- LCAGSSYRHMR (SEQ ID NO:16)
	Rat Annexin II	145-VYKEMYKTDLEK (SEQ ID NO:17)
	Rat P450c26/25	424- VLCTYVVSRR (SEQ ID NO:18)
	Strept. cholesterol oxidase	420- VSLYLAIK (SEQ ID NO:19)
	Mouse cholesterol 7"-monooxygenase	167-VTEGMYAFCYR (SEQ ID NO:20)
30	Nocardia cholesterol dehydrogenase	91- VTEAYRQR (SEQ ID NO:21)
	Human Bile-Salt-Activated-Lipase	226- LSPYNKGLIR (SEQ ID NO:22)
	Rabbit acyl-CoA cholesterol acyltransferase	96- VVDYIDEGR (SEQ ID NO:23)

The presence of the cholesterol interaction/ recognition consensus sequence in the proteins listed in Table 1 signifies the likelihood that the proteins interact with cholesterol and provides insight into how these protein accomplish their functions in concert with cholesterol and how to alter these functions. For example, in the case of annexin which is a calcium-phospholipid/cholesterol actin binding protein important for various cell functions, i.e. from maintaining cell membrane shape and stability to facilitating the internalization of plasma membrane components, altering the cholesterol recognition/interaction site of annexin may result in destabilization and collapse of the cytoskeleton. This could be a useful method for targetting and destroying tumor cell growth.

After reviewing the description of the present invention, it would be within the skill of a person with ordinary skill in the art to design mutant proteins in which the cholesterol recognition/interaction consensus sequence has been altered such that the protein's ability to interact/recognize cholesterol is reduced, increased, or abolished. Experiments on these mutants will further provide treatments for diseases associated with an altered, reduced, or increased function of these proteins, or even perhaps a method for targeting specific proteins through their ability to interact with cholesterol for altering the cholesterol recognition/interaction function for a desired purpose.

After reviewing the present disclosure, it will be evident to a person with ordinary skill in the art that it is possible to determine whether or not a protein interacts with cholesterol or recognizes

cholesterol. This knowledge will provide insight into how a specific protein, whether known or yet to be discovered, functions or is regulated.

In another embodiment, the present invention
5 provides a nucleotide sequence which encodes the amino acid sequences, or peptides described above. The nucleotides corresponding to codons specifying the amino acids of the consensus sequence described above are known to people in the art. These sequences can
10 include for example:

Leu Asn Tyr Cys Val Trp Arg (SEQ ID NO:7).

The generation of nucleic acid molecules encoding the cholesterol interaction/recognition amino acid consensus sequence(s) described above is routine for a
15 person with ordinary skill in the art. Nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be
20 double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand. Also included are chemically modified and substituted nucleic acids,
25 e.g., those which incorporate modified nucleotide bases or which incorporate a labelling group.

The nucleic acid may be isolated. By "isolated" is meant a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For
30 example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid

molecules according to the present invention further include such molecules produced synthetically.

Such isolated nucleic acid molecules are useful for probes for detecting a gene which contains this
5 consensus sequence in a biological sample, for instance, by PCR, Southern blot, Northern blot, or other form of hybridization analysis as described in Molecular Cloning: A Laboratory Manual, 2nd edition, Sambrook, J. Fritsch, E.F. and Maniatis T., eds., Cold
10 Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) or DNA Cloning, Volumes I and II (D. N. Glover ed., 1985) or Current Protocols in Molecular Biology Ausubel, F. M. et al. (Eds.) John Wiley & Sons, Inc. for general cloning methods). The entire
15 disclosures of documents cited in this application are incorporated in their entirety by reference thereto. Typical nucleic acid probes may be prepared from the consensus amino acid sequences. In particular, probes may be prepared based upon segments of the amino acid
20 sequence which possess relatively low levels of degeneracy, i.e., few or one possible nucleic acid sequences which encode therefor. These probes may further comprise a detectable group for easy identification and location of complementary
25 sequences.

cDNA or genomic libraries of various types may be screened for new alleles or related sequences using the above probes. Phage or plasmid libraries may be used.

30 In addition to comprising a segment which encodes the peptides having the consensus sequence of the present invention, the nucleic acid of the present invention may also comprise a segment encoding a

heterologous protein, such that the gene is expressed to produce the two proteins as a fusion protein.

Nucleic acid molecules of the present invention which encode the amino acid consensus sequence of the present invention may include, but are not limited to those encoding the amino acid sequence of the consensus by itself; and additional coding sequences which code for additional amino acids, such as those which provide additional functionalities. Thus, the sequences encoding the consensus sequence may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide.

In addition to their use as probes, the nucleic acids of the present invention may also be used in the preparation of the peptides having the consensus amino acid sequence of the present invention.

DNA encoding the peptides with the consensus amino acid sequence of the present invention will typically be incorporated into DNA constructs capable of introduction to and expression in an *in vitro* cell culture. Often, the nucleic acids of the present invention may be used to produce a suitable recombinant host cell. Specifically, DNA constructs will be suitable for replication in a unicellular host, such as bacteria, e.g., *E. coli*, but may also be intended for introduction into a cultured mammalian, plant, insect or other eukaryotic cell lines. DNA constructs prepared for introduction into bacteria or yeast will typically include a replication system recognized by the host, the intended DNA segment encoding the desired polypeptide, transcriptional and translational initiation and termination regulatory sequences operably linked to the polypeptide encoding segment. A DNA segment is operably linked when it is

placed into a functional relationship with another DNA segment. For example, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence; DNA for a signal
5 sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide. Generally, DNA sequences that are operably linked are contiguous, and in the case of a signal sequence both
10 contiguous and in reading phase. However, enhancers need not be contiguous with the coding sequences whose transcription they control. Linking is accomplished by ligation at convenient restriction sites or adapters or linkers inserted in lieu thereof. The selection of
15 an appropriate promoter sequence will generally depend upon the host cell selected for the expression of the DNA segment. Examples of suitable promoter sequences include prokaryotic, and eukaryotic promoters well known in the art and include promoters such as the
20 *trp*, *lac* and phage promoters, tRNA promoters and glycolytic enzyme promoters, T7 promoter, T3 promoter, SP6 promoter, SV40 promoter, CMV promoter and MoMLV LTR are known and available. See Sambrook *et al.*, 1989, *supra*. The transcriptional regulatory sequences
25 will typically include a heterologous enhancer or promoter which is recognized by the host.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial
30 plasmids, bacteriophage, yeast episomes, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof such as
35 cosmids and phagemids.

Conveniently available expression vectors which include the replication system and transcriptional and translational regulatory sequences together with the insertion site for the consensus sequence peptide encoding segment may be employed. Among vectors preferred for use in bacteria include pBS vectors, Phagescript vectors, Bluescript vectors commercially available. Among preferred eukaryotic vectors are pSV2CAT, pWLNEO, available from Stratagene; and pSVK2, pMSG available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan such as pET vectors, such as pET15b, pZeoSV2, pCMV and the like. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al. and in Metzger et al., *Nature* 334, 31-36, 1988. For example, where an insect host cell is selected as the host cell of choice to express the polypeptide, the cDNA encoding the polypeptides of the invention may be cloned into a baculovirus expression vector (pV-IKS). The recombinant baculovirus may then be used to transfect a suitable insect host cell, e.g. SF9 cells, which may then express the polypeptide. See, e.g. D.D. Morrison et al., *Cell* 58, 649-657, 1989, M.D. Summers and G.E. Smith, A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Station, College Station, Tex., 1987.

The vectors containing the DNA segments of interest, e.g. those encoding the peptides comprising the consensus sequence of the present invention, can be transferred into the host cell by well known methods, which may vary depending upon the type of host used. For example, calcium chloride transfection is commonly used for prokaryotic cells, whereas

calcium phosphate treatment may be used for other hosts. Introduction of the construct into the host cell can be effected by DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. See Davis et al. Basic Methods in Molecular Biology, 1986. The term "transformed cell" as used herein, includes the progeny of originally transformed cells.

10 In another embodiment, the present invention provides a cell stably transfected with a vector comprising the cholesterol interaction/recognition consensus sequence of the present invention. The term "transfected" as used herein refers to cell having
15 undergone the process of introduction of nucleic acid or a nucleic acid vector into a cell. Various methods of transfecting a cell are possible including microinjection, CaPO_4 precipitation, lipofection (liposome fusion), electroporation and use of a gene
20 gun. The term "stable" as used herein refers to the introduction of a gene into the chromosome of the targeted cell where it integrates and becomes a permanent component of the genetic material in that cell. A transfected cell containing a vector having a
25 peptide comprising the consensus sequence may only be transiently transfected, resulting in transient expression of the peptide. The term "transient" as used herein relates to the introduction of a gene into a cell to express a cholesterol consensus containing
30 peptide, where the introduced DNA is not integrated into the host cell genome and is accordingly eliminated from the host cell over a period of time. Transient expression relates to the expression of a product during a period of transient transfection. An

episomal transfection is a variant of stable transfection in which the introduced gene is not incorporated in the host cell chromosomes but rather is replicated as an extrachromosomal element. This
5 can lead to apparently stable transfection of the characteristics of a cell.

A cell may be transfected with a vector containing a selectable marker or cotransfected with a second vector containing the desired selectable
10 marker. This selectable marker is used to select those cells which have become transfected. Types of selectable markers which may be used are well known to those of ordinary skill in the art.

In another embodiment, there is provided a
15 transfected cell wherein a peptide comprising the cholesterol consensus sequence of the present invention is expressed as a cell surface peptide. By "cell surface" peptide is meant a peptide which wholly or partially spans the cell membrane, and which is
20 exposed on the surface of the cell. The peptide comprising the cholesterol consensus sequence of the present invention can be expressed as a secreted peptide. By "secreted peptide" is meant a peptide which is not associated with the cell membrane, but
25 rather is intracellularly processed for secretion into the extracellular environment or other cellular compartment.

The peptide comprising the consensus amino acid sequence of the present invention can be recovered and
30 purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction
35 chromatography, affinity chromatography,

hydroxylapatite chromatography, lectin chromatography, and high performance liquid chromatography ("HPLC") is employed for purification. Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells.

10 An additional preferred embodiment provides for a transgenic animal containing a cholesterol interaction/recognition consensus sequence nucleic acid. The consensus sequence can also be presented on a vector alone, or as part of a gene. By "transgenic animal" is meant an animal whose genome contains an additional copy or copies of the desired gene from the same species or another species introduced by genetic manipulation or cloning techniques, as described herein and as known in the art. The transgenic animal can include the resulting animal in which the vector has been inserted into the embryo from which the animal developed or any progeny of that animal. The term "progeny" as used herein includes direct progeny of the transgenic animal as well as any progeny of succeeding progeny. Thus, one skilled in the art will readily recognize that if two different transgenic animals have been made each utilizing a different gene or genes and they are mated, the possibility exists that some of the resulting progeny will contain two or more introduced genes. One skilled in the art will readily recognize that by controlling the matings, transgenic animals containing multiple introduced genes can be made. This transgenic animal is useful in screening compounds for their pharmacological

effects on cholesterol interaction, recognition, uptake and metabolism in the transgenic animal.

In another embodiment, the present invention provides a transgenic insect. Studies have shown that
5 PBR is found in insects and is homologous to the mammalian PBR. Therefore, using methods known in the art, it is possible to introduce a mutation in the genomic PBR sequence as described above such that the ability of PBR to recognize or interact with
10 cholesterol is abolished or reduced, or increase steroid production by introducing an additional cholesterol interaction/recognition sequence. The ability to control the production of steroids in insects by controlling the uptake of cholesterol
15 through PBR represents a powerful method for controlling insect reproduction.

In another embodiment of the present invention is provided a transgenic plant comprising in its genome a nucleic acid encoding the cholesterol
20 interaction/recognition sequence or an altered cholesterol interaction/recognition sequence wherein cholesterol interaction is reduced, abolished or increased. The cholesterol interaction/recognition sequence can be delivered alone or as part of a gene,
25 or in a vector alone or as part of a gene. Evidence indicates that the PBR receptor in plants functions similarly to the mammalian PBR and is homologous in sequence. Therefore, it is another aspect of the invention to provide a transgenic plant comprising in
30 its genome a genetic construct comprising a nucleic acid encoding PBR with an altered cholesterol recognition/interaction sequence wherein the ability of PBR to interact with cholesterol and channel it into the cell is reduced, abolished, or increased
35 thereby resulting in a reduced, abolished or increased

uptake of cholesterol in the cell, and/or reduced, abolished, or increased production of steroids in the transgenic plant. This method may offer an opportunity to regulate cholesterol levels in plants, an well as specific plant steroids such as cardenolides, etc. required for nutrition or any other purpose. Research has demonstrated that transgenic plants are capable of passing on the inserted genes to their progeny by normal Mendelian inheritance (Christou et al., 1990, *Trends in Biotechnol.* 8, 145-151). All such progeny plants which inherit the inserted genetic construct are also transgenic plants as the term is used here.

The production of transgenic plants is known in the art. Please see for example, U.S. Patent No. 5,869,720 to John, M.E. issued on February 9, 1999, and U.S. Patent No. 5,859,347 to Brown et al. issued on January 12, 1999. The disclosures of both patents are hereby incorporated in their entirety by reference thereto.

Briefly, the nucleic acid desired can be inserted into a suitable plant transformation vector for transformation into the desired plant species. Suitable plant transformation vectors include those derived from a *Ti* plasmid of *Agrobacterium tumefaciens*. A plant transformation vector preferably includes all of the necessary elements needed for transformation of plants, or plant cells. Specific plant tissues can be targeted by using promoters specific for expression in fruit, fiber, root, etc... Promoters used can be inducible by plant hormone such as ecdysone, by antibiotic, such as tetracycline, by changes in temperature, such as heat shock elements. Typical plant transformation vectors comprise selectable marker genes, one or both of the T-DNA

borders, cloning sites, appropriate bacterial genes to facilitate identification of transconjugates, broad host-range replication and mobilization functions and other elements as desired.

5 Transformation of plant cells may be effected by delivery of a transformation vector or of free DNA by use of a particle gun which comprises high velocity microprojectiles coated with the vector into plant tissue. Selection of transformed plant cells and
10 regeneration into whole plants may be carried out using conventional procedures. Other transformation techniques capable of inserting the desired gene or nucleic acid into plant cells may be used, such as electroporation or chemicals that increase free nucleic
15 acid uptake. Illustrative examples of methods suitable for regenerating transgenic plants are : corn (Fromm et al., 1990, *Bio/Technology* 8:833-839; and Gordon-Kamm et al., 1990 *The Plant Cell* 2:603-618); rice (Wang et al., 1988, *Plant Mol. Biol.* 11:433-439)
20 and wheat (Vasil et al., *Bio/Technology* 8:743-747).

In one aspect, the transformed or transfected cells are useful for screening agents or drugs which alter the ability of polypeptides comprising the consensus sequence and mutants of said sequence to
25 interact, recognize or bind to cholesterol or its derivatives. An *in vitro* system for determining whether a compound, drug, or agent is an agonist or antagonist of the ability of the polypeptide comprising the consensus sequence of the present
30 invention to interact with cholesterol can be designed. A polypeptide comprising the consensus sequence of the present invention can be incubated along with cholesterol under conditions, i.e. salts, pH, lipid, ions, where interaction between cholesterol
35 and the peptide comprising the consensus sequence

occurs, . The complex may then be incubated with a test compound. Interaction between the polypeptide and cholesterol may then be measured. An increase or decrease in the level of interaction between the polypeptide and cholesterol in response to a particular compound would indicate that the compound is an agonist or antagonist of that binding, respectively.

The same test can be administered on cells which express a polypeptide comprising the cholesterol interaction/recognition consensus sequence. The pH and temperature which are most effective for cholesterol interaction are preferably used but it is possible to replicate the conditions found in a diseased cell for testing the effect of a particular drug with respect to a proposed therapy for the disease.

A test compound may be a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal cells or tissues.

For many of the methods of the present invention, the polypeptides and nucleic acids of the invention may be covalently attached or linked to a detectable group to facilitate screening and detection. Useful detectable groups or labels, are generally well known in the art. For example, a detectable group may be a radiolabel, such as, ^{125}I , ^{32}P , or ^{35}S , or a fluorescent or chemiluminescent group. Alternatively, the detectable group, may be a substrate, cofactor, inhibitor, affinity ligand, antibody binding epitope tag, or an enzyme which is capable of being assayed. Suitable enzymes include, e.g., horseradish peroxidase, luciferase, or another readily assayable

enzymes. These enzyme groups may be attached to the polypeptide comprising the consensus sequence by chemical means or expressed as a fusion protein as already described.

5 In another embodiment, the present invention provides a method for detecting presence, absence, increase or decrease of cholesterol or a derivative of cholesterol in a biological sample. This can be done in several ways, for example, in some instances, it
10 may be useful to immobilize the polypeptide comprising the consensus sequence upon a solid support, e.g., a microtiter well, or nitrocellulose membrane. The sample to be assayed is exposed to the immobilized polypeptide, and the amount of cholesterol-bound
15 polypeptide is measured.

Alternatively, the present invention provides a method for detecting the presence of a polypeptide comprising the consensus sequence of the present invention by coating on a solid support cholesterol or
20 a derivative of cholesterol capable of interacting with the peptide comprising the cholesterol interaction/recognition sequence, and exposing said cholesterol or its derivative to a sample thought to contain a polypeptide(s) comprising the consensus
25 sequence of the present invention and detecting cholesterol-bound polypeptide. For detection of an increase or decrease in the level of cholesterol, or a derivative of cholesterol, comparison to appropriate controls may be necessary.

30 The solid support can include agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose, polystyrene, filter paper, nitrocellulose, ion exchange resins, plastic films, glass beads, polyaminemethylvinylether maleic acid copolymer, amino
35 acid copolymer, ethylene-maleic acid copolymer, nylon,

silk, etc. The support may be in the form of, e.g., a test tube, microtiter plate, beads, test strips, or the like. The reaction of the solid support with either cholesterol or the polypeptide may be carried
5 out by methods well known in the art.

By "biological sample" is intended any biological sample obtained from an animal, cell line, tissue culture, plant, insect, or other source which may contain cholesterol or its derivatives, or cholesterol
10 interacting/recognizing polypeptides mRNA or DNA. Biological samples include body fluids (such as saliva, blood, plasma, urine, mucus, synovial fluid, etc.) tissues (such as muscle, skin, and cartilage) and any other biological source suspected of
15 containing cholesterol or derivatives of cholesterol, or cholesterol binding polypeptides or nucleic acids. Methods for obtaining biological samples such as tissue are well known in the art.

In another embodiment, the present invention
20 provides a method for identifying whether or not a protein recognizes or interacts with cholesterol or cholesterol derivatives said method comprising identifying the presence or absence of the above-mentioned cholesterol recognition/
25 interaction consensus by analyzing the cholesterol interaction/ recognition amino acid sequence of the unknown protein as discussed in the examples below. The presence of the amino acid sequence of the present invention is an indication of the likelihood that the
30 protein recognizes/interacts with cholesterol or a cholesterol derivative.

In another embodiment, the present invention provides a method for conferring to a molecule the ability to interact with cholesterol or a cholesterol
35 derivative which is recognized by the cholesterol

recognition/interaction sequence. Since the consensus sequence for interaction/recognition of cholesterol has been elucidated, it is now possible to introduce into a molecule or polypeptide, natural or synthetic, a cholesterol recognition/interaction sequence such that the protein or polypeptide, natural or synthetic, is now able to recognize/interact with cholesterol or a cholesterol derivative. The consensus sequence can be introduced at the DNA or RNA level, by inserting the nucleotide sequence encoding the consensus sequence into a part of the gene for said molecule, such that the consensus sequence is translated along with the molecule and together they form a fusion protein. Preferably, the consensus sequence is inserted at the amino or carboxy terminal of the gene such that secondary and tertiary folding of the gene product does not inhibit the interaction of the consensus sequence with cholesterol. The fusion protein can be tested for its ability to interact/recognize or bind cholesterol or a cholesterol derivative.. These fusion proteins can be used for therapy or diagnostics. The molecule into which the cholesterol interaction/recognition sequence is desired can be natural or synthetic.

In yet another embodiment, the present invention provides a molecule which blocks the cholesterol interaction/recognition ability of a peptide comprising the cholesterol recognition/interaction consensus described above to recognize/interact with cholesterol. The molecule can be an antibody, a peptide, or drug. Antibody production using peptides is well known in the art, see, e.g., Sutcliffe, *et al.* *Science* 219, 660-666, 1983; Wilson *et al.*, *Cell* 37, 767-778, 1984, and Bittle *et al.*, *J. Gen. Virol.* 66, 2357-2354, 1985. As used herein, the term "antibody"

(AB) or "monoclonal antibody (Mab) is meant to include intact molecules, single chain whole antibodies, and antibody fragments. Antibody fragments of the present invention include Fab and F(ab')₂ and other fragments including single chain Fvs (scFv) and disulfide-linked Fvs (sdFv). Also included in the present invention are chimeric and humanized monoclonal antibodies and polyclonal antibodies specific for the polypeptides of the present invention. The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing a polypeptide of the present invention or an antigenic fragment thereof can be administered to an animal in order to induce the production of sera containing polyclonal antibodies. Monoclonal antibodies can be prepared using hybridoma technology known in the art see, e.g., Harlow et al., Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory Press 2nd ed., 1988). Additionally, antibodies capable of binding to a polypeptide antigen of the present invention may be produced through the use of anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and that, therefore, it is possible to obtain an antibody which binds to a second antibody.

Additionally, the cholesterol interaction/recognition peptide may be useful in modeling small molecules which interfere with cholesterol binding in vivo. In particular, the structure of the cholesterol interaction/recognition domain from the known amino acid sequence and the 3-dimensional structure, which may be determined by x-ray crystallographic methods known in the art, may be applied in generating synthetic analogs, cholesterol derivatives, and mimics of the particular cholesterol

interaction/recognition domain. Synthetic elements may be pieced together based upon their analogy to the structural and chemical aspects of the cholesterol interaction/recognition domain. Such mimics, analogs, and derivatives may be used in blocking or inhibiting specific functions resulting from cholesterol binding to a peptide or gene product. These functions can vary since a role for cholesterol has been implicated in cell signaling, cell proliferation, gene regulation, cytoskeletal anchoring and stability, neural transmission, fertility, stress, diabetes, stroke, to name a few, and may thus be useful as therapeutic treatments according to the methods described herein.

The present invention includes both bio chips and biosensor comprising polynucleotides, polypeptides, and antibodies of the present invention and methods of their use. Bio chips comprising arrays of polynucleotides, polypeptides, and antibodies of the present invention may be used to detect the presence of same in a biological or environmental sample and to diagnose an animal, including human. Methods and particular uses of the polynucleotides of the present invention to detect the same using bio chip technology include those known in the art and those of: U.S. Patent Nos. 5510270, 5677195, 5607646 and World Patent Nos. WO97/10365, WO97/43447, each incorporated herein in their entireties.

Biosensors using the polynucleotides, polypeptides and antibodies of the present invention may also be used to detect, monitor, and diagnose. Methods and particular uses of the polynucleotides, polypeptides and antibodies of the present invention using biosensors include those known in the art and those of: U.S. Patent Nos. 5721102, 5658732, and World

Patent Nos. WO97/35011, WO97/20203, each incorporated herein in their entireties.

In another embodiment, the present invention relates to a method for altering the cholesterol binding ability of molecules which contain the cholesterol recognition/interaction consensus described above, comprising altering said site such that cholesterol recognition is reduced, eliminated, or increased. Increased cholesterol recognition occurs when the cholesterol pocket is perfectly formed or by introducing into a molecule a new or additional cholesterol recognition/interaction sequence. We have been able to produce PBR with reduced or no ability to recognize and interact with cholesterol by modifying the cholesterol recognition/interaction site of PBR, for example by changing amino acid 153 from tyrosine to serine, or by changing amino acid 155 from arginine to leucine. These modifications are expected to produce similar results in species which express PBR, and on proteins other than PBR which contain the consensus sequence. Therefore, it would be possible to alter the cholesterol recognition/interaction ability of a polypeptide comprising the peptide consensus sequence of the present invention by changing the tyrosine in the consensus sequence to serine or other amino acid with no charge, for example, or changing the consensus sequence arginine to leucine or any other amino acid without a charge.

In this application is described the consensus sequence for a cholesterol interaction/recognition site first discovered on PBR. In addition to the presence of PBR in the mitochondrial membrane, PBR is present in the plasma membrane (Oke, B. O. et al. 1992, *Mol. Cell. Endocr.* 87:R1-R6; Garnier, M. et al. 1993, *Endocrinology* 132:444) and in the nuclear

membrane (Hardwick, M. et al. 1999, *Cancer Research* 59:831-842). Results reported in this application indicate that the carboxy terminal of the receptor is responsible for the interaction and subsequent uptake
5 of cholesterol. Cholesterol is released from the receptor once a ligand binds to the amino terminal portion of the receptor. In the case of the mitochondria, the cholesterol is released into the inner mitochondrial membrane where it is available for
10 interaction with P450scc and is cleaved to produce pregnenolone in steroidogenic cells. Pregnenolone is the precursor of steroids.

Therefore, the present invention relates to a method for increasing cholesterol in the membrane of a
15 cell by inhibiting its release from the receptor, PBR. Alternatively, a method is provided for decreasing the presence of cholesterol inside the cell by introducing an agent which inhibits PBR ligand binding to PBR thereby inhibiting the release of cholesterol from
20 PBR.

In another embodiment, the present invention relates to a method for increasing the presence of cholesterol inside the cell by introducing a PBR ligand such that the cholesterol is released from PBR.

25 In steroidogenic cells, an increase in the release of cholesterol from PBR due to ligand binding would result in an increase in pregnenolone production. Therefore, a method is provided for increasing pregnenolone production in a steroidogenic
30 cell, comprising providing a PBR ligand to said cell such that PBR releases cholesterol which is then available for cleavage into pregnenolone. The peptide can be targeted to specific steroid producing cells by complexing it with leutinizing hormone for targeting
35 to the testes, with follicle stimulating hormone for

targeting to the ovaries, and with ACTH for targeting to the adrenal. Conversely, a method for decreasing pregnenolone production in a steroidogenic cell, comprising providing a PBR ligand inhibitor to said
5 cell such that release of PBR bound cholesterol is inhibited and therefore cleavage of the cholesterol to pregnenolone is inhibited.

Since pregnenolone is the precursor for steroid production, a method for increasing steroid production
10 in a steroidogenic cell, comprising providing a PBR ligand to said cell such that PBR releases cholesterol which is then available for cleavage into pregnenolone and used as the precursor of all steroids. Similarly, a method for reducing steroid production in a
15 steroidogenic cell, comprising providing an agent which inhibits PBR ligand binding to PBR such that PBR-bound cholesterol is not released and said cholesterol is not cleaved into pregnenolone, the precursor of all steroids. This method is useful for
20 reducing disease symptoms resulting from increased production of steroids such as stress and Cushing's disease.

In addition to the above described uses, the polypeptides and nucleic acids of the present
25 invention may also be used in therapeutic applications for the treatment of human or non-human mammalian patients.

The nucleic acid encoding the polypeptide comprising the cholesterol interaction sequence of the
30 present invention can be used as a cholesterol acceptor in order to regulate the amount of cholesterol, or cholesterol derivatives, in a tissue or blood. The nucleic acid is administered alone or as part of a vector such that it is translated, and
35 the resulting polypeptide is capable of interacting

with/binding to cholesterol. The nucleic acid sequence encoding a peptide comprising the cholesterol interaction/recognition consensus sequence can be administered prophylactically, or to patients having a disease or condition characterized by an elevated plasma cholesterol level. By "elevated level" is meant a higher level relative to what is normally found in the plasma. Administration can be by exogenous delivery of the nucleic acid as naked DNA, DNA associated with specific carriers, or in a nucleic acid expression vector to a desired tissue by means of an appropriate delivery vehicle, e.g. a liposome, by use of iontophoresis, electroporation and other pharmacologically approved methods of delivery. Some methods of delivery may include: encapsulation in liposomes, transduction by retroviral vectors, localization to nuclear compartment utilizing nuclear targeting site found on most nuclear proteins, transfection of cells *ex vivo* with subsequent reimplantation or administration of the transfected cells, a DNA transporter system. Intravenous administration with a drug carrier designed to increase the circulation half-life of the nucleic acid encoding the cholesterol interaction/recognition sequence can be used. Additionally, the treatment of any disorder may comprise gene therapy techniques involving the cholesterol interaction/recognition domain sequence or a mutation or alteration of the cholesterol interaction/recognition domain sequence. Strategies for gene therapy are reviewed in Friedman, *Science* 244, 1275, 1989.

Drug delivery vehicles are effective for both systemic and topical administration. They can be designed to serve as a slow release reservoir, or to deliver their contents directly to the target cell.

An advantage of using direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs which would otherwise
5 be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

The nucleic acid sequence encoding a cholesterol
10 recognition/interaction sequence may also be systemically administered. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic
15 absorption include: intravenous, intramuscular, subcutaneous, intraperitoneal, intranasal, intrathecal and ophthalmic. A gene gun may also be utilized. The dosage will depend upon the disease indication and the route of administration but should be between 1-1000
20 ug/kg of body weight/day. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon disease delivery vehicle and efficacy data from clinical trials.

25 The nucleic acid comprising the cholesterol interaction/recognition consensus sequence may be administered utilizing an *in vivo* approach whereby the nucleic acid will be administered directly to an animal by intravenous injection, intramuscular
30 injection, or by catheterization and direct delivery of the nucleic acid via the blood vessels and directed to a target organ by using tissue-specific promoters.

The polypeptides of the present invention may be used to inhibit or block uptake of cholesterol into
35 cells by competing for cholesterol. These methods may

generally be used in the treatment of a variety of diseases resulting from an increase in cholesterol in the cell or in the plasma, or in screening compounds effective for such treatment. Cholesterol is required
5 for normal cell growth and proper membrane structure and function. Unregulated accumulation of cholesterol is cytotoxic and a failure to maintain cholesterol homeostasis results in a number of pathological states (See: Subcellular Biochemistry vol. 28, *Cholesterol: its Functions and Metabolism in Biology and Medicine*.
10 Bittman, Robert (Ed), Plenum Press, N.Y.). Specific disorders include gallstones, atherosclerosis, Niemann-Pick C, Sitosterolemia, Dystrophy, Tumor proliferation (tumorigenesis), Schnyder's corneal
15 crystalline dystrophy. Brain disorders include cholesterol metabolism and Alzheimer's disease, Tellurium toxicity, Smith-Lemli-Opitz syndrome, myelinization, developmental abnormalities and demyelization : Charcot-Marie-Tooth disease;
20 Pelizaeus-Merzbacher disease, Multiple sclerosis, SLA, to name a few. Alternatively, the methods and compositions may be useful as prophylactic treatment, or in screening for compounds effective in prophylactic treatments.

25 In another aspect of the invention, the peptide comprising the cholesterol recognition/interaction consensus sequence of the present invention can be used to deliver, when needed, cholesterol, or a cholesterol derivative which binds the cholesterol
30 recognition/interaction sequence of the present invention. The peptide can be complexed to cholesterol prior to administration, thereby avoiding the nonspecific attachment of cholesterol to other factors such as albumin for example.

The quantities of reagents necessary for effective therapy, also referred to herein as an "effective amount", or "therapeutically effective amount", will depend upon many different factors, including means of administration, target site, Physiological state of the patient and other mdicants administered. Thus, treatment doses will need to be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Generally, therapeutically effective amounts of the polypeptide comprising the cholesterol interaction/recognition domain of the present invention will be from about 0.0001 to about 100 mg/kg, and more usually, from about 0.001 to about 0.1 mg/kg of the subject's body weight. Various considerations are described, e.g., in Gilman et al., (Eds.), Goodman and Gilman's: The Pharmacological Basis of Therapeutics, (8th ed., 1990), Pergamon Press, and Remington's Pharmaceutical Sciences (7th ed., 1985) Mack Publishing Co., Easton, Pa. Methods of administration, also discussed in the above references, include, e.g., oral, intravenous, intraperitoneal or intramuscular administration, and local administration, including topical, transdermal diffusion and aerosol administration, for therapeutic, and/or prophylactic treatment. The active agent will generally be administered in a composition additionally comprising a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds

described in, e.g., the Merck Index, Merck and Co.,
Rahway, N.J.

By "subject" is meant fish, animals, including
plants, insects, monkeys, apes, cats, dogs, birds,
5 cows, pigs, mice, horses, rabbits and humans.

Constituents of pharmaceutical compositions, in
addition to the active agents, include those generally
known in the art for the various administration
methods used. For example, oral forms generally
10 include powders, tablets, pills, capsules, lozenges
and liquids. Similarly, intravenous, intraperitoneal
or intramuscular formulations will generally be
dissolved or suspended in a pharmaceutically acceptable
carrier, e.g., water, buffered water, saline, and the
15 like. Additionally, these compositions may include
additional constituents which may be required to
approximate physiological conditions, such as pH
adjusting and buffering agents, toxicity adjusting
agents, wetting agents and the like. For solid
20 compositions, conventional nontoxic solid carriers may
be used which include, e.g., pharmaceutical grades of
mannitol, lactose, starch, magnesium stearate, sodium
saccharin, talcum, cellulose, glucose, sucrose,
magnesium carbonate, and the like.

25 Administration may also be carried out by way of
a controlled release composition or device, whereby a
slow release of the active ingredient allows
continuous administration over a longer period of
time.

30 The following MATERIALS AND METHODS were used in
the examples that follow.

MA-10 Leydig cells- MA-10 mouse Leydig tumor
cells were maintained as we previously described
(Papadopoulos, V. et al. 1990, *J. Biol. Chem.*
35 265:3772-3779). For the cholesterol uptake assays,

mitochondria were isolated as we described (Papadopoulos *et al.* 1990, *supra*; Krueger and Papadopoulos, 1990, *supra*) and resuspended in buffer A (250mM Sucrose, 20mM KCl, 15mM triethanolamine hydrochloride [pH7.0], 10mM K₃PO₄ and 10mM MgCl₂) at 1mg/ml total protein concentration (Leaver, H. A. and Boyd G. S. 1981, *J. Endocrinol.* 91:123-133). Mitochondria were then incubated with [1,2-³H]-cholesterol (0.127 uCi/100 nmol) in 0.3 ml buffer A at 37 °C (or the indicated temperature) for the indicated time period. At the end of the incubation, steroids were extracted and ³H-pregnenolone formed was isolated by thin layer chromatography and quantified (Amri, H. *et al.* 1996, *Endocrinology* 137:5707-5718).

15

Construction of the mouse PBR expression vector (pET15bPBR) and Expression of Recombinant PBR in bacteria- The pET system (Novagen, Madison, WI) was used to express the MA-10 mouse PBR (mPBR) recombinant protein. The insert containing full length coding sequence as well as the NdeI and XhoI site extensions at the 5' and 3' ends were generated by PCR using the following primers: ATATATACATATGCCTGAATCCTGGGTG (SEQ ID NO:24) and ATACTCGAGTGGGTGCCTTCACTCTG (SEQ ID NO:25), respectively. The MA-10 full length PBR cDNA (Garnier, M. *et al.* 1994, *Mol. Pharm.* 45:201-211) was used as template. This mPBR fragment was inserted into pET15b vector and linearized with NdeI and XhoI downstream of the T7lac promoter. Recombinant mPBR expression vector was used to transform the BL21(DE3) *Escherichia coli* strain (Novagen) where the expression of recombinant mPBR protein was induced by 1mM isopropyl-1-thiol- β -D-galactopyranoside (IPTG). PBR protein expression was monitored by SDS-PAGE followed by Coomassie Blue staining or immunoblot analysis

using anti-PBR antiserum (Amri et al. 1996, *supra*). Binding specificity of the IPTG-induced PBR in *E. coli* was determined in binding studies where specific binding of ^3H -PK 11195 (1.0 nM) was measured in the presence of the indicated concentrations of the indicated ligands.

^3H -Cholesterol uptake by *E. coli* cells was examined using the indicated concentrations of control or IPTG-treated transformed bacteria incubated in the presence of 6.7 nM ^3H -cholesterol (50.0 Ci/mmol) for 60 min at 37°C. Specific cholesterol uptake is defined as IPTG-induced minus basal values. *E. coli* protoplasts were prepared from cells grown in LB at 37°C to the logarithmic phase of growth and centrifuged at 10,000g for 5 min. The cells were washed twice in 10mM Tris-HCl buffer [pH8.0] and the pellet was resuspended in a solution containing 20%(w/w) sucrose and 0.1M Tris-HCl [pH8.0]. The cells were then suspended in 1ml of buffer per 10 OD₄₅₀ and mixed. Within 1 min lysozyme was added from a 2mg/ml stock solution in distilled water to a final concentration of 100 ug/ml at 37°C, and stirring was continued for the next 12 min. The cell suspension was diluted 1:10 with 0.1M Na₂EDTA prewarmed to 37°C. Continuous stirring and slow dilution over 2.5 min prevented cell lysis. More than 99% of the cells became spherical within 10 minutes (Weiss, R. L. 1978, *Meth. Cell Biol.*20:141-147). Protoplasts (100 ug in 0.3 ml final volume) were then incubated in the presence of 6.7 nM ^3H -cholesterol (50.0 Ci/mmol) for 60 min at 37°C for cholesterol uptake assays. ^3H -Cholesterol-labeled membranes of IPTG-induced transformed bacteria, incubated with 30 nM ^3H -cholesterol, were treated with PK 11195 or clonazepam

and the ^3H -cholesterol released was quantified by liquid scintillation spectrometry.

Site-directed Mutagenesis- Mutations were performed using the QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). Briefly, miniprep pET-PBR plasmid dsDNA was used as template. Synthetic oligonucleotide primer pairs containing A147T, Y153S, R155L point mutations and PBR deletions, $\Delta 5-20$, $\Delta 41-51$, $\Delta 108-119$, $\Delta 120-133$, $\Delta 141-152$, $\Delta 153-169$, each complementary to the opposing strand of the vector, were extended during temperature cycling by *pfu* DNA polymerase. Upon incorporation of the oligonucleotide primers, mutated plasmids containing staggered nicks were generated. After temperature cycling, the products were treated with *Dpn* I is used to digest the parental DNA template and select for the generated mutation. The nicked vector DNAs containing the desired mutations were then transformed into *E. coli*. The mutated plasmids were prepared by ABI Prism Miniprep Kit. The generated mutations and deletions were confirmed by sequencing using the ABI Prism Dye Terminator Cycle Sequencing ready reaction kit (Perkin Elmer Applied Biosystems, Foster City, CA). DNA sequencing was performed at the Lombardi Cancer Center Sequencing Core Facility (Georgetown University).

Radioligand binding assays- ^3H -1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide (PK 11195) binding studies were performed as we previously described (Papadopoulos, V. et al. 1990, *J. Biol. Chem.* 265: 3772-3779; Garnier, M. et al. 1994, *Mol. Pharm.* 45:201-211). The dissociation constant (K_d) and the

number of binding sites (Bmax) were determined by
Scatchard plot analysis of the data using the LIGAND
program (Munson, P. J. and Rodbard, D. 1980, *Anal.*
Biochem. 107:220-239).

5

Protein measurement- Microgram amounts of
protein were quantified using the dye-binding assay of
Bradford (Bradford, M. M. 1976, *Anal. Biochem.* 72:248-
254) using bovine serum albumin as the standard.

10

Statistics- The results shown represent the means
± S.D. or S.E.M. from 2 to 6 independent experiments.
Statistical analysis was performed by ANOVA followed
by the *Student-Newman-Keuls* test or the *Dunnett*
multiple comparisons test using the Instat (v.2.04)
package from GraphPad, Inc. (San Diego, CA).

15

Example 1

A three-dimensional model of human PBR was built
using molecular dynamics simulations and shown to
accommodate a cholesterol molecule within its five
helices (Bernassau, J. M. et al. 1993, *J. Mol. Graph.*
11:236-245). Despite differences in the primary amino
acid sequence between the human and mouse PBR, similar
data was obtained when the mouse 18 kDa PBR protein
was submitted to the same analysis (Papadopoulos, V.
1996, *supra*), further suggesting that PBR may function
as a channel for Cholesterol. In order to test this
hypothetical model we used two cell model systems:
(i) the MA-10 Leydig cells, a steroidogenic cell model
which expresses high levels of PBR (~40 pmol/mg
protein; Papadopoulos, V. et al. 1990, *J. Biol. Chem.*
265:3772-3779), and as all eukaryotic cells contains
endogenous cholesterol; and (ii) the *E.coli* DE3 cells

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25
30

which do not express PBR (this study), do not have endogenous cholesterol (Moat, A.G. and Foster, J.W. 1995, Microbial Physiology. Wiley-Liss, New York.) and do not form steroids. Thus, using these cell models we attempted to correlate PBR expression with the cholesterol transport function. In addition, we used a method with radiolabeled cholesterol (Leaver, H. A. and Boyd G. S., 1981, *J. Endocrinol.* 91:123-133) to quantify cholesterol movement. This method allows for the distinction between the exogenous cholesterol, permitting an easy quantification, and direct measurement of the pregnenolone formed, in the case of the steroidogenic cells where cholesterol transported to IMM is cleaved by the P450scc to generate pregnenolone. Data shown in Fig. 1 validate the use of this method. Fig. 1 shows that in the steroidogenic Leydig cells, ³H-cholesterol uptake by the mitochondria and transport from OMM to IMM were stimulated by specific PBR ligands resulting in increased ³H-pregnenolone formation. These data are in agreement with previous findings in all steroid synthesizing cell types of the body (Papadopoulos, V. 1993, *Endocr. Rev.* 14:222-240; Papadopoulos, V. 1998, *Proc. Soc. Exp. Biol. Med.* 217:130-142). Moreover, a similar PBR-dependent cholesterol transport mechanism from OMM to IMM was recently identified in liver mitochondria (Tsankova, V. et al., 1995, *Eur. J. Pharm.* 294:601-607). Cholesterol detoxification from the periphery by the IMM sterol-27-hydroxylase (Tsankova et al., 1995, *supra*). Interestingly, the rate of cholesterol uptake and intramitochondrial transport to IMM, in response to PBR ligand activation, was identical (0.9 nmol/mg protein/min) for adrenal (Krueger, K.E. and

Papadopoulos, V. 1990, *J. Biol. Chem.* 265:15015-15022) and liver (Tsankova et al. 1995, *supra*) mitochondria, suggesting that a similar PBR-mediated cholesterol transport mechanism is operative in both steroidogenic
5 and non-steroidogenic tissues.

As noted above a bacterium is a model system without endogenous cholesterol (Moat and Foster, 1995, *supra*). In addition, bacteria do not express PBR protein (Fig. 2A) and ligand binding (not shown)
10 although the presence of a PBR homologous protein, the tryptophan-rich-sensory-protein *tspO* (also called *crtK*), involved in carotenoid biosynthesis in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* photosynthetic bacteria, has been reported (Yeliseev, A.A. and Kaplan S. 1995, *J. Biol. Chem.* 270:21167-
15 21175). *Escherichia coli* were transfected with mouse PBR cDNA in a pET vector. Addition of IPTG to transfected bacteria resulted in the expression of the 18 kDa PBR protein (Fig. 2A) and ligand binding (Fig. 2B; $K_d=1.1$ nM and $B_{max}=0.23$ pmol/mg protein) with
20 similar pharmacological characteristics to that previously described for PBR (Papadopoulos, 1993, *supra*; Papadopoulos, V. et al. 1990, *supra*) (Fig. 2C). IPTG-induced PBR expression resulted in a protein (Fig. 3A), time (Fig. 3B), and temperature-
25 dependent uptake of radiolabeled cholesterol (Fig. 3B). This cholesterol uptake was maintained when protoplasts were prepared indicating that PBR resides in the internal bacterial plasma membrane (data not
30 shown). The uptake of cholesterol could not be blocked by energy poisons (DeGrella, R. F. and Simoni, R. d., 1982, *J. Biol. Chem.* 257: 14256-14262) (Fig. 3C). In addition, it was specific for cholesterol since no uptake of other radiolabeled steroid could be
35 seen (Fig. 3D), and could not be saturated at the

concentrations of radiolabeled cholesterol used (Fig. 3E), suggesting that PBR functions as a channel for cholesterol rather than a cholesterol-binding protein, which is in agreement with the modeling studies (Bernassau, et al., 1993, *supra*; Papadopoulos, V. 1996, *supra*). When IPTG-induced, cholesterol-loaded, bacterial membranes were treated with PK 11195, cholesterol was liberated from the membranes (Fig. 3F), suggesting that cholesterol captured by PBR is released upon ligand binding. Thus, PBR serves a channel-like or a port function where cholesterol can enter and reside stored within the membrane without interacting with the lipid or protein components of the lipid bilayer. This may also be the way by which the mitochondria sort between the steroidogenic pool of cholesterol from the cholesterol component of the membrane. Thus, PBR ligands control the opening/release state of the channel, mediating cholesterol movement across membranes. In addition to the PBR drug ligands, the polypeptide diazepam binding inhibitor (DBI) and porphyrins (Papadopoulos, V. 1993, *supra*) have been identified as naturally occurring endogenous ligands. It should be noted that at present we couldn't exclude the possibility that PBR functions as a flippase or a transporter.

In support of the data presented herein, targeted disruption of the PBR gene in steroidogenic cells resulted in inhibition of cholesterol transport to IMM and arrest of steroid biosynthesis (Papadopoulos, V. et al. 1997, *J. Biol. Chem.* 272:32129-32135). Transfecting PBR mutant Leydig cells with the PBR cDNA rescued steroidogenesis and demonstrated the obligatory role of PBR in cholesterol transport (Papadopoulos, V. 1996, *supra*).

Example 2

PBR is an 18 kDa hydrophobic protein with five putative transmembrane domains located in the OMM. As a first step in defining the regions of the receptor involved in the interaction with the drug ligand and cholesterol, we constructed mutant PBRs with the deletions indicated in the left panel of Fig. 4. The location of the five transmembrane regions of the receptor (I to V) is also shown in Fig. 4. It should be noted that the amino-terminus of mitochondrial membrane proteins is directed towards the inside of the organelle whereas the carboxy-terminus is in the cytoplasmic side. The right panel of Fig. 4 shows the effect of deletion of specific amino acid sequences on PBR ligand binding and cholesterol uptake examined in the bacterial system described above. Deletion of amino acid sequences 5-20 and 41-51 in the amino-terminus of the receptor decreased by 30-45% the ability of PBR to bind the ligand PK 11195. Our results are in agreement with previous studies on human PBR expressed in yeast (Farges, R. *et al.*, 1994, *Mol. Pharm.* 46:1160-1167), although in those studies deletion of the amino-terminus of human PBR completely abolished the ability of the receptor to bind PK 11195. Deletion of amino acids 120-133 in the fourth transmembrane domain also decreased PBR ligand binding by 45%. Smaller decrease (25%) of PK 11195 binding was also seen when the regions 141-152 and 153-169 were deleted. These results suggest that although the amino-terminus of the receptor may confer the ability to bind drug ligands, such as the isoquinoline PK 11195, amino acid sequences in the fourth transmembrane domain may participate in the formation of the ligand binding site. It should be noted that

deletions affecting PK 11195 ligand binding did not had a major effect on the ability of the recombinant receptor expressed in bacterial membranes to take up radiolabeled cholesterol.

5 Fig. 4 also shows that deletion of amino acids 153-169 in the carboxy-terminus of PBR had a dramatic effect on the ability of the molecule to take up ³H-cholesterol when expressed in bacteria (70% decrease). This result suggests that the cytoplasmic carboxy-
10 terminal domain of the receptor is responsible for the interaction and subsequent uptake of cholesterol. In an effort to identify specific amino acids in PBR responsible for the interaction with cholesterol we undertook site-directed mutagenesis studies in the
15 carboxy-terminal region.

Example 3

Recent studies by Pikuleva *et al.* (1995, *Arch. Biochem. Biophys.* 322:189-197) with another protein
20 that interacts with cholesterol, the enzyme P450scc, indicated that a Tyrosine in the active site of the P450scc interacts with the side chain of cholesterol. Aligning the P450scc active site amino acid sequence with the carboxy-terminus of PBR indicated that there
25 might be a common amino acid consensus pattern in these two molecules recognizing cholesterol (Table I). This consensus pattern is composed of a neutral and hydrophobic amino acid (Z), such as Leucine or Valine, a neutral and polar amino acid (Y), such as Tyrosine,
30 and a basic amino acid (Q), such as Arginine or Lysine. One to five different amino acids may be placed between these three coding amino acids. Thus, the proposed consensus pattern is **-Z-(X)₀₋₅-Y-(X)₀₋₅-Q-**. Leucine or Valine will interact with the
35 hydrophobic side chain of cholesterol and Tyrosine

will interact with the polar 3'OH-group of cholesterol, whereas the Arginine or Lysine may help create a pocket. This hypothesis was tested (Fig. 5). Replacement of Y153 by Serine or R156 by Leucine completely abolished the ability of PBR to take up radiolabeled cholesterol. Mutation and replacement of A147 with Threonine, did not affect cholesterol uptake by bacteria expressing the mutated receptor. Fig. 5 also shows that the wild-type and mutated recombinant receptor proteins were expressed at equal levels upon IPTG induction.

Example 4

In an effort to see whether this putative cholesterol recognition/interaction amino acid consensus pattern is present in other molecules shown or suggested to interact with cholesterol, such as the as apolipoprotein A-1 (Boyle, T. P. and Marotti, K.R., 1992, Gene 117, 243-247), caveolin (Murata, M. et al. 1995, Proc. Natl. Aca. Sci. USA 92, 10339-10343), DBI (Papadopoulos, V. 1998, Proc. Soc. Exp. Biol. Med. 217, 130-142), steroidogenesis acute regulatory protein (StAR) (Stocco, D. M. and Clark, B. J. 1996, Endocr. Rev. 17, 221-244), hedgehog protein (Porter, J. A. et al. 1996, Science 274, 255-259), cytochrome P450 C26/25 (Su, P. et al. 1990, DNA Cell Biol. 9-657-667), annexin II (Harder, T. et al. 1997, Mol. Biol. Cell 8, 533-545), sterol carrier protein-2 (Colles, S. M. et al. 1995, Lipids 30, 795-803), cholesterol 7 α -monooxygenase (Kai, M. et al. 1995, Lipid Res. 36, 367-374), cholesterol oxidase (Ishizaki, T. et al. 1991, J. Bacteriol. 171, 596-601), cholesterol dehydrogenase (Horinouchi, S. et al. 1991, Appl. Environ. Microbiol. 57, 1386-1393), bile-salt-

activated lipase precursor (cholesterol esterase) (Nilsson, J. et al. 1990, *Eur. J. Biochem.* 192, 543-550), and acyl-CoA cholesterol acyltransferase (Pape, M.E. et al. 1995, *J. Lipid Res.* 36, 823-838) we looked
5 for the presence of the cholesterol recognition/interaction amino acid consensus pattern - **Z-(X)₀₋₅-Y-(X)₀₋₅-Q-** in these proteins. Table I shows that all these proteins, with the exception of sterol carrier protein-2, contain this amino acid consensus
10 pattern. Proteins such as rat skeletal muscle alpha-actin, non-muscle and smooth muscle myosin light chain did not contain this cholesterol recognition/interaction consensus pattern. However, given any tyrosine there is a reasonably high
15 probability that this consensus amino acid sequence will be found in many proteins. Indeed, a motif search through the various gene data banks indicated that this amino acid consensus pattern is present in various proteins. This is not surprising since it is
20 known that the cholesterol/protein interaction plays a role not only in cholesterol transport and/or storage but also in protein stability, folding, and/or localization. Thus, it is possible that only in some proteins this consensus sequence will be functional.
25 The strength and specificity of the interaction of a protein containing this consensus amino acid sequence with cholesterol may be due either to the presence of a certain microenvironment, or the location of the consensus sequence within the protein, or a specific
30 conformation of the protein that allows the use of this amino acid sequence. In the latter case, it is also possible that the consensus sequence identified represents only a portion, maybe the core, of a larger motif to be identified.

Of special interest to steroidogenesis is the observation that the cholesterol recognition/interaction amino acid consensus pattern was found in the polypeptide DBI (Papadopoulos, V. 1993, *supra*; Papadopoulos, V. 1998, *supra*) and the precursor StAR protein (Stocco, D. M. and Clark, B. J. 1996, *Endocr. Rev.* 17, 221-244). In search of a cytosolic steroidogenesis-stimulating factor, a protein was purified shown to be identical to DBI (Papadopoulos, V. 1993, *supra*). DBI was originally purified from brain by monitoring its ability to displace diazepam from its recognition sites in synaptosomes. DBI is also identical to the acyl-CoA-binding protein (Knudsen, J. et al., 1993, *Mol. Cell. Biochem.* 123:129-138). Purified DBI was shown to stimulate intramitochondrial cholesterol transport and increase pregnenolone formation by isolated mitochondria (Papadopoulos, V., 1998, *supra*). Later on, it was demonstrated that this action of DBI was mediated by PBR (Papadopoulos, V. 1993, *supra*; Papadopoulos, V. 1998, *supra*). In addition DBI was shown to increase cholesterol loading onto isolated P450scc (Brown, A. S. and Hall, P. F., 1991 *Biochem. Biophys. Res. Commun.* 180:609-614). Thus, the identification of the cholesterol recognition/interaction amino acid consensus pattern in DBI may help understand its role in steroidogenesis and its direct effect on P450scc. Interestingly, we showed in the past that the naturally occurring processing product of DBI, the triakontatetrapeptide TTN (DBI₁₇₋₅₀), but not the octadecaneuropeptide ODN (DBI₃₃₋₅₀), was able to mimic the effect of DBI on mitochondrial steroidogenesis (Papadopoulos, V. et al. 1991, *Endocrinology* 129:1481-1488). The finding that in DBI the cholesterol recognition/interaction amino

acid consensus pattern is located in the amino acid sequence 25 to 32 (Table I) may now explain this result. It should be also noted that the cholesterol recognition/interaction amino acid consensus pattern
5 is found in the middle of the acyl-CoA-binding protein signature domain (amino acids 19 to 37) important in forming the acyl-CoA-binding site (Knudsen *et al.* 1993, *supra*).

STAR has been found in gonadal and adrenal cells,
10 where it is newly synthesized in response to trophic hormones, as a cytoplasmic precursor protein of 37 kDa targeted to mitochondria (Stocco and Clark, 1996 *supra*). STAR synthesis in Leydig cells begins 60 min after addition of the hormone and then parallels the
15 capacity of the cells to produce steroids in response to tropic hormones (Stocco and Clark, 1996 *supra*; Clark, B. J. *et al.*, 1995, *Mol. Endocr.* 9:1346-1355). The 37 kDa STAR precursor further undergoes cleavage to produce the 30 kDa mitochondrial "mature" STAR
20 protein and its phosphorylated counterpart (Stocco and Clark, 1996 *supra*). This protein processing is believed to occur at the level of the outer/inner mitochondrial membrane contact sites and it has been proposed to be responsible for cholesterol transport
25 from outer to inner mitochondrial membrane (Stocco and Clark, 1996 *supra*). Considering that the cholesterol recognition/interaction amino acid consensus pattern is found in the amino-terminus of the STAR precursor protein, which is removed from the mature protein, it
30 is possible that the function of the precursor STAR protein is to shuttle cholesterol from intracellular stores to the outer mitochondrial membrane.

In conclusion, the results presented herein demonstrate that PBR may have a channel-like function
35 for cholesterol in the OMM. The steroidogenic pool of

cholesterol, coming from various intracellular sources, is recognized by the cholesterol recognition/interaction amino acid consensus pattern - **Z-(X)₀₋₅-Y-(X)₀₋₅-Q-** present in the carboxy-terminus of PBR in the OMM. This pool of cholesterol enters in the OMM at the PBR sites where it remains without mixing with other membrane components. Ligand binding to the receptor induces the release of this cholesterol. Considering that PBR has been shown to be associated with the voltage-dependent anion channel (Papadopoulos, V. 1998, *supra*), found in the outer/inner mitochondrial membrane contact sites, the released cholesterol could now directly access the P450scc in the IMM where it will be cleaved to pregnenolone, precursor of all steroids.

In addition to being a precursor for steroid hormone synthesis, cholesterol is an essential structural element of cellular membranes and a precursor for the synthesis of bile acids and lipoproteins. Mammalian cells obtain cholesterol by internalization of low-density lipoproteins or by *de novo* synthesis in the endoplasmic reticulum. The subcellular distribution of cholesterol suggests that cholesterol is trafficked and incorporated quickly from the sites of acquisition to the target membrane (Liscum, L and Underwood, K. W. 1995, *J. Biol. Chem.* 270:15433-15446). Thus, a tissue and cell specific cholesterol homeostasis is achieved. Considering the widespread occurrence of PBR and its tissue and cell specific subcellular localization (Papadopoulos, V. 1993, *supra*; Papadopoulos, V. 1998, *supra*), these results suggest a more general role for PBR in intracellular cholesterol transport and compartmentalization.

What is claimed is:

1. A cholesterol recognition/interaction amino acid consensus sequence comprising

5 Z-(X)₀₋₅-Y-(X)₀₋₅-Q

wherein Z is a neutral hydrophobic amino acid, Y is a neutral polar amino acid, Q is a basic amino acid and X is any amino acid.

10 2. The cholesterol recognition/interaction amino acid consensus sequence of claim 1 wherein Z is Leucine or Valine.

15 3. The cholesterol recognition/interaction amino acid consensus sequence of claim 1 wherein Y is Tyrosine.

20 4. The cholesterol recognition/interaction amino acid consensus sequence of claim 1 wherein Q is Arginine or Lysine.

25 5. The cholesterol recognition/interaction amino acid consensus sequence of claim 1 wherein
 (i) Z is leucine or Valine;
 (ii) Q is Arginine or Lysine; and
 (iii) Y is Tyrosine.

30 6. The cholesterol recognition/interaction amino acid consensus sequence of claim 1 wherein X is one amino acid.

7. The cholesterol recognition/interaction amino acid consensus sequence of claim 1 wherein X is two amino acids.

8. The cholesterol recognition/interaction amino acid consensus sequence of claim 1 wherein X is 1-3 amino acids.

5

9. A nucleic acid molecule encoding the consensus sequence of claim 1.

10. A nucleic acid molecule comprising:

10

(i) a vector; and

(ii) the nucleic acid molecule of comprising a cholesterol interaction/recognition consensus of claim 9.

15

11. The nucleic acid molecule according to claim 10 wherein said vector is a prokaryotic vector.

12. The nucleic acid molecule according to claim 11 wherein said vector is an expression vector.

20

13. The nucleic acid molecule according to claim 10 wherein said vector is a eukaryotic vector.

14. The nucleic acid molecule according to claim 13 wherein said vector is an expression vector.

15. The nucleic acid molecule according to claim 13 wherein said vector is useful for expression in plants.

30

16. A host cell transformed with the nucleic acid molecule of claim 10.

17. The host cell of claim 16 wherein said host cell is prokaryotic.

35

18. The host cell of claim 16 wherein said host cell is eukaryotic.

5 19. The host cell of claim 16 wherein said host is a plant cell.

20. A peptide comprising a cholesterol interaction/recognition sequence according to claim 1.
10

21. A method for detecting whether or not a protein recognizes cholesterol comprising identifying in the amino acid sequence or the nucleic acid sequence of said protein the presence or absence of a cholesterol recognition/interaction consensus sequence
15 according to claim 1 wherein the presence of the consensus sequence indicates possible interaction/recognition of the protein with cholesterol.
20

22. A method for conferring cholesterol recognition/interaction to a molecule comprising introducing into said molecule a cholesterol recognition/interaction sequence according to claim 1
25 such that said sequence is expressed and said molecule interacts with cholesterol.

23. A method for reducing serum cholesterol in a subject, said method comprising introducing into said
30 subject a nucleic acid comprising the cholesterol interaction/recognition consensus sequence according to claim 1 such that it is expressed and is able to interact with cholesterol.

24. A method for delivering cholesterol to a subject comprising administering a peptide comprising the cholesterol interaction/recognition consensus sequence according to claim 1 complexed with
5 cholesterol in a pharmaceutically acceptable amount, in a pharmaceutically acceptable diluent.

25. A method for detecting an increase or decrease of cholesterol in a biological sample
10 comprising
immobilizing a polypeptide comprising the cholesterol interaction/recognition consensus sequence according to claim 1 on a solid support rendering an immobilized polypeptide,
15 exposing the sample to the immobilized polypeptide, and
measuring the amount of cholesterol-bound polypeptide wherein when comparing to a standard, an increase or decrease over the standard can be
20 determined.

26. A method for screening agents or drugs which are agonists or antagonist of interaction between peptides comprising the cholesterol recognition/
25 interaction consensus sequence according to claim 1 and cholesterol comprising
exposing a polypeptide comprising the consensus sequence of the present invention to cholesterol under conditions where interaction between cholesterol and
30 the peptide occurs forming a peptide/cholesterol complex
incubating the complex with a test compound
measuring an increase or decrease in the level of interaction between the polypeptide and cholesterol in
35 response to the test compound where an increase in



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interaction would indicate that the test compound is an agonist and a decrease in interaction would indicate that the test compound is an antagonist of peptide/cholesterol binding.

5

27. A molecule which blocks cholesterol interaction with the cholesterol recognition/interaction consensus sequence according to claim 1.

10

28. The molecule according to claim 27 wherein said molecule is selected from the group consisting of: a peptide, a drug, and an antibody.

15

29. A method for reducing the cholesterol binding ability of peptide which comprise the cholesterol recognition/interaction consensus sequence according to claim 1 comprising

20

modifying Y from a tyrosine to a serine, or
modifying Q from an arginine to a leucine.

25

30. A peripheral-type benzodiazepine receptor wherein the cholesterol recognition/interaction function of said receptor is reduced according to the method of claim 29.

30

31. A peripheral-type benzodiazepine receptor unable to recognize/interact with cholesterol said receptor comprising a deletion comprising a cholesterol interaction/recognition sequence of said receptor.

35

31. A method for reducing disease symptoms in a subject resulting from an increase in cholesterol, said method comprising administering to said subject a



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nucleic acid encoding a peptide comprising a
cholesterol recognition/interaction consensus sequence
such that said nucleic acid is expressed and said
peptide is produced in a therapeutically effective
5 amount.

32. The method of claim 31 wherein said
administration is by microspheres.

10 33. A transgenic plant comprising a nucleic acid
encoding a peripheral-type benzodiazepine receptor
according to claim 30.

15 34. A transgenic plant comprising a nucleic acid
encoding a peripheral-type benzodiazepine receptor
according to claim 31.

20 35. A transgenic plant comprising a nucleic acid
encoding a peripheral-type benzodiazepine receptor
operably linked to an inducible promoter.

36. The transgenic plant according to claim 35
wherein said promoter is inducible any of the
following conditions: heat, administration of
25 antibiotic, administration of plant hormone.



FIG. 1

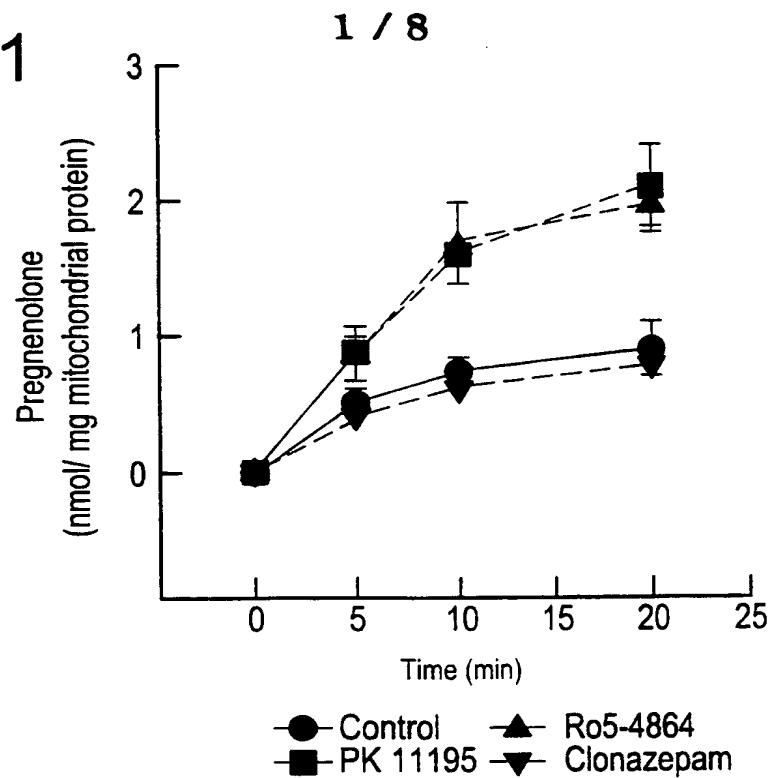
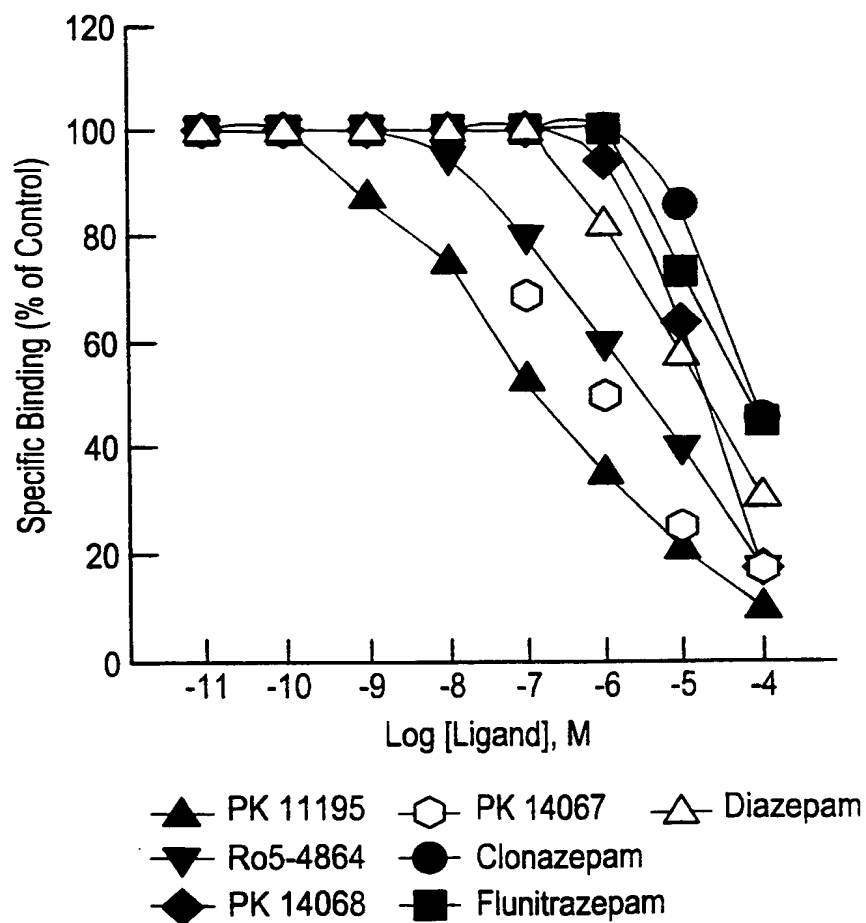


FIG. 2B





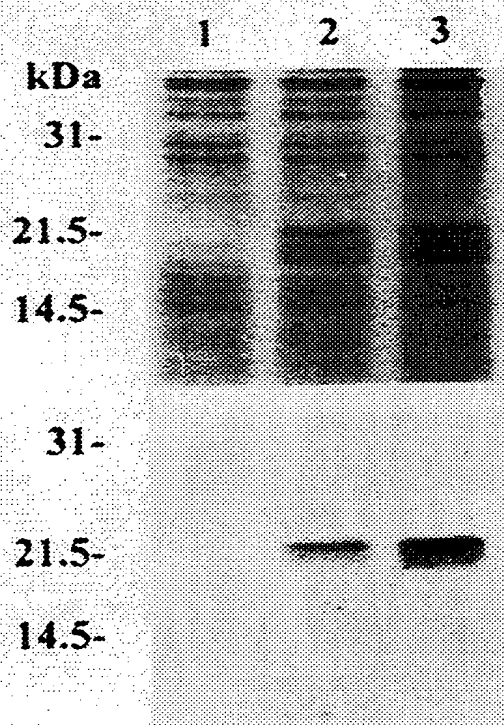


FIG.2A



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FIG. 2C

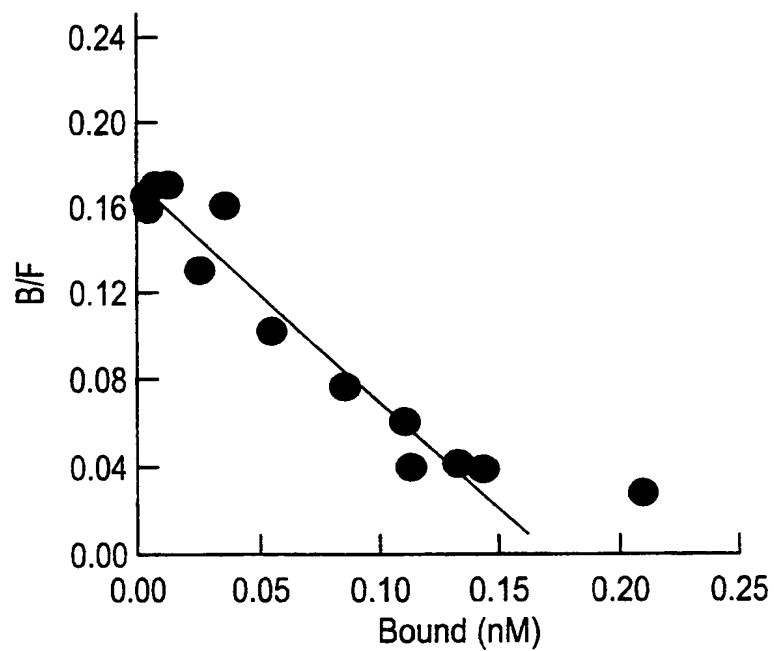
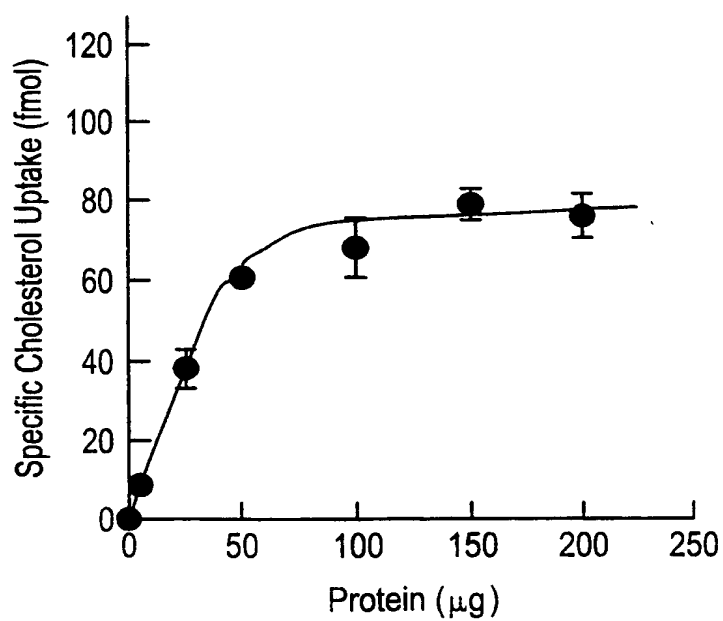


FIG. 3A





4 / 8

FIG. 3B

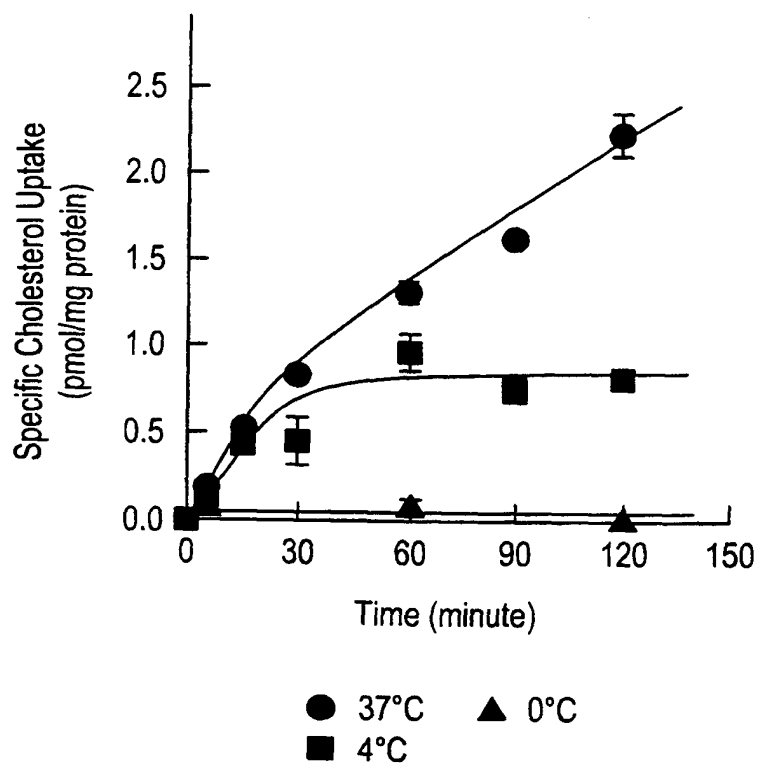
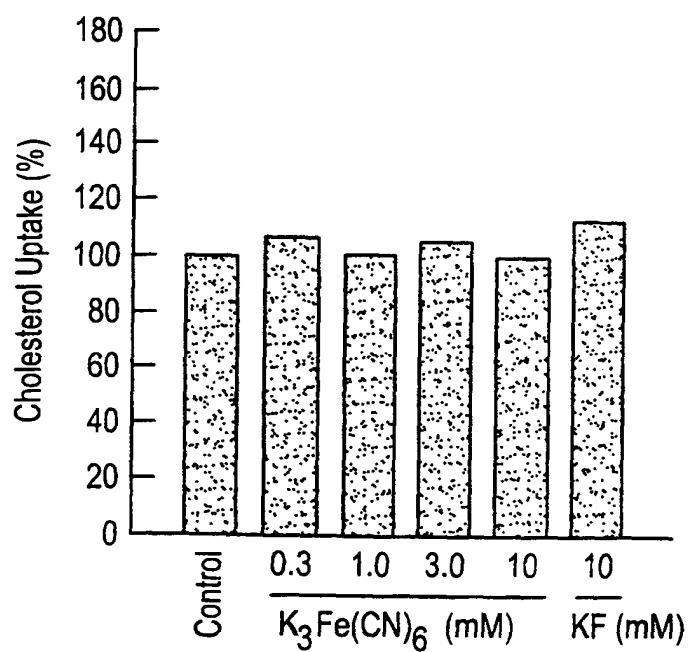


FIG. 3C





5 / 8

FIG. 3D

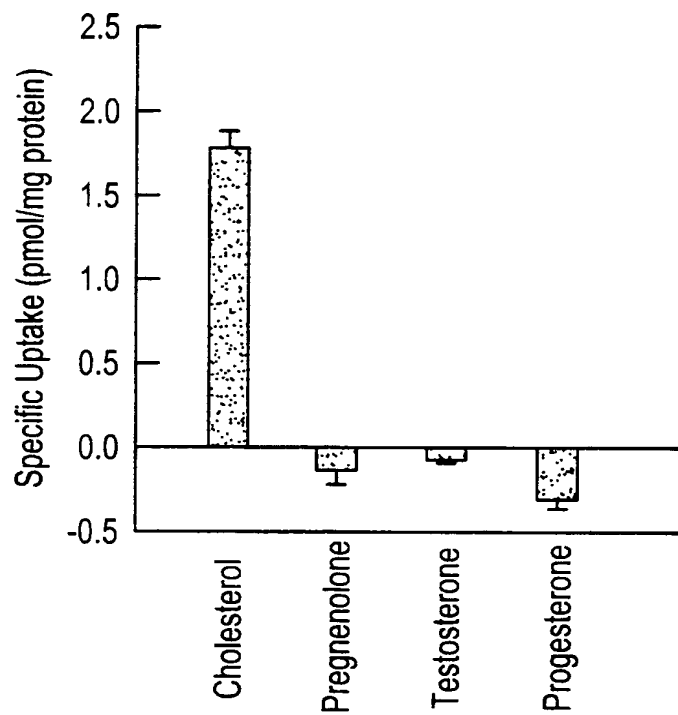
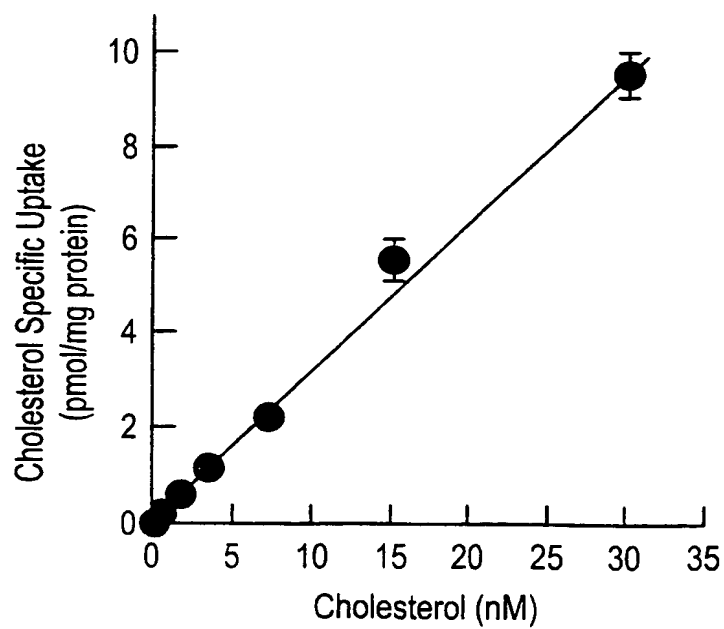


FIG. 3E





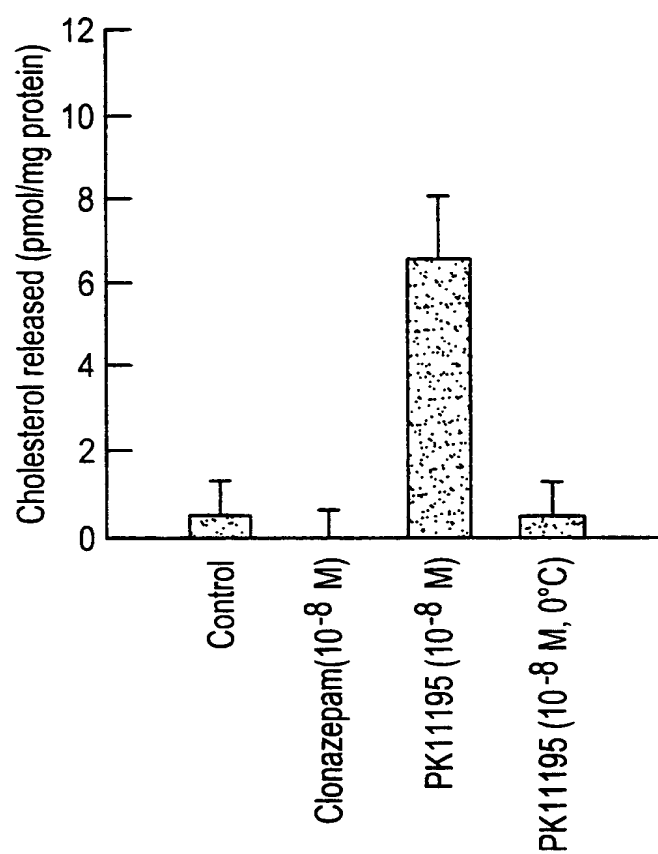
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FIG. 3F





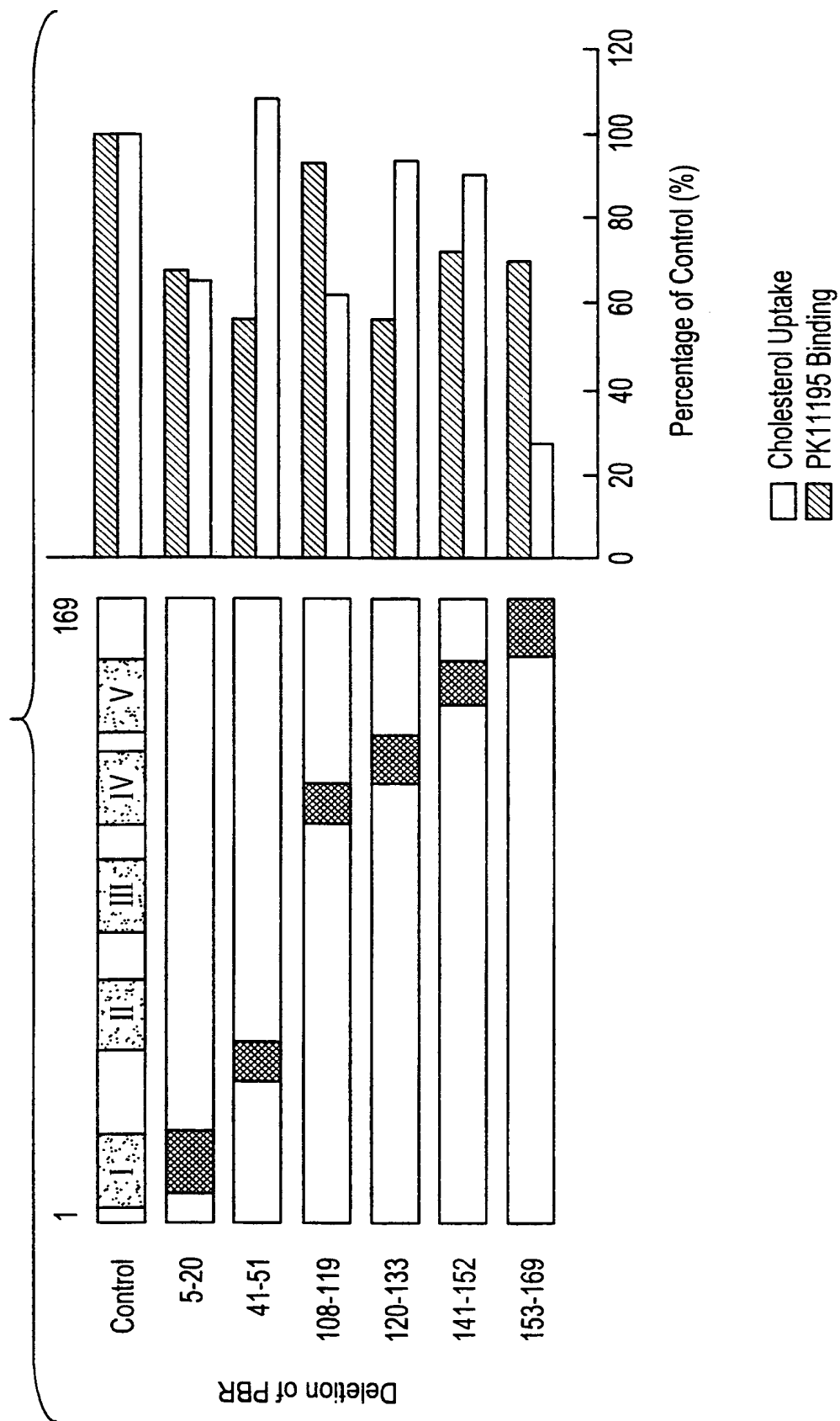
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FIG. 4





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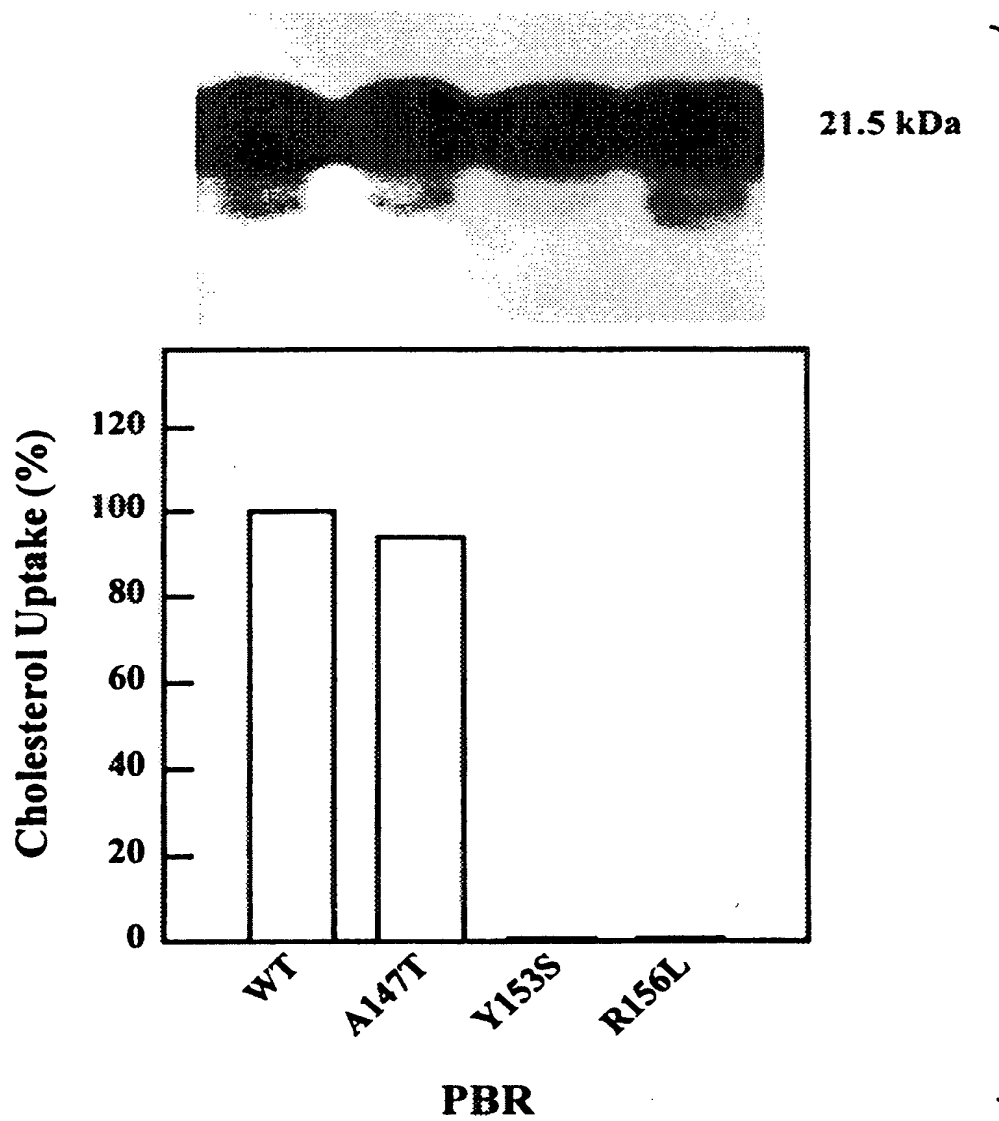


FIG.5



This demand must be filed directly with the competent International Preliminary Examining Authority, or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two letter code of the Authority may be indicated by the applicant: IPEA

PCT
Demand CHAPTER II

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

-----For International Preliminary Examination Authority use only-----

Identification of IPEA

Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's agent's file reference: 009/112/SAP

International Application No.: PCT/US99/05853

International Filing date (day/month/year): 12 March 1999 (12/03/99)

(Earliest Priority date (day/month/year): 12 March 1998 (12/03/98)

Title of invention: Cholesterol Recognition Sequence

Box No. II APPLICANT(S)

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)

Name: Georgetown University Medical Center

Address: Georgetown University Medical Center
4000 Reservoir Road, N.W.
Washington, D.C. 20007

Telephone No.: 202-687-1390

Facsimile No:

State (i.e. country of nationality): US

State (i.e. country of residence): US

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)

Name: Papadopoulos, Vassilios

Address: 15417 Peach Leaf Drive
North Potomac, Maryland 20878

Telephone No.:

Facsimile No:

State (i.e. country of nationality): US

State (i.e. country of residence): US

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)

Name: Li, Hua

Address: 1319 Fort Myer Drive, Apt. 102
Arlington, Virginia

Telephone No.:

Facsimile No:

State (i.e. country of nationality): US

State (i.e. country of residence): US

[] Further applicants are indicated on a continuation sheet.

International application No. PCT/US99/05853

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative
and

☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and Address (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country)

Name: Pratt, Sana A.

Address: 10821 Hillbrooke Lane
Potomac, Maryland 20854
US

Telephone No.: 301-294-9171

Facsimile No.: 301-294-7357

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV STATEMENT CONCERNING AMENDMENTS

The applicant wishes the International Preliminary Examining Authority*

- (i) ☒ to start the international preliminary examination on the basis of the international application as originally filed.
- (ii) ☐ to take into account the amendments under Article 34 of
 - ☐ the description (amendments attached).
 - ☐ the claims (amendments attached).
 - ☐ the drawings (amendments attached).
- (iii) ☐ to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).
- (iv) ☐ to disregard any amendments of the claims made under Article 19 and to consider them as reversed.
- (v) ☐ to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). (This check box may be marked only where the time limit under Article 19 has not yet expired).

*Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES

☒ The applicant hereby elects all eligible States (that is, all States which have been designated and which are bound by Chapter II of the PCT) except-----

(If the applicant does not wish to elect certain eligible states, the name(s) or country code(s) of those States must be indicated above.)

Box No. VI CHECK LIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:


	For International Preliminary Examining Authority use only	
	Received	not received
1. amendments under Article 34		
description : <input type="checkbox"/> sheets	<input type="checkbox"/>	<input type="checkbox"/>
claims : <input type="checkbox"/> sheets	<input type="checkbox"/>	<input type="checkbox"/>
drawings : <input type="checkbox"/> sheets	<input type="checkbox"/>	<input type="checkbox"/>
2. letter accompanying amendments under Article 34 : <input type="checkbox"/> sheets	<input type="checkbox"/>	<input type="checkbox"/>
3. copy of amendments under Article 19 : <input type="checkbox"/> sheets	<input type="checkbox"/>	<input type="checkbox"/>
4. copy of statement under Article 19 : <input type="checkbox"/> sheets	<input type="checkbox"/>	<input type="checkbox"/>
5. other (specify): _____ : <input type="checkbox"/> sheets	<input type="checkbox"/>	<input type="checkbox"/>

The demand is also accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☒ fee calculation sheet
5. ☐ other (specify): _____

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (is such capacity is not obvious from reading the demand).


Sana A. Pratt,
Applicants' Agent
U.S. Agent No.: 39,441

-----For International Preliminary Examining Authority use only-----

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply.

☐ The applicant has been informed accordingly.

4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.

5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

-----For International Bureau use only-----

Demand received from IPEA on:

Fee Calculation Sheet

Annex to the Demand for International preliminary examination

International Application No.:
PCT/US99/05853

For International Preliminary Examining Authority use only

Applicant's or Agent's
file reference 009/112/SAP

Date Stamp of the IPEA

Applicant : Georgetown University Medical Center

CALCULATION OF PRESCRIBED FEES

1. Preliminary examination fee..... 750.00 (P)

2. Handling Fee..... 162.00 (H)

3. Total of prescribed fees

Add the amounts entered at P and H
and enter total at TOTAL

TOTAL: U.S. \$912.00

MODE OF PAYMENT

☒ authorization to charge deposit account (see below) () bank draft
() coupons () cheque () cash () postal money order
() revenue stamps () other specify:

DEPOSIT ACCOUNT AUTHORIZATION (this mode of payment may not be available at all Offices)

The IPEA/ US ☒ is hereby authorized to charge the total fees indicated to deposit account NO: 07-1075.

☒ (this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit) is hereby authorized to charge any deficiency or credit any overpayment in the total fees indication above to my deposit account NO: 07-1075.

Signature

Sana A. Pratt
Sana A. Pratt

U.S. Reg. NO. 39,441

Date

13 Sept. 1999

**PCT
REQUEST**

For receiving Office use only

International Application no.

International Filing Date: 12 March 1999
(12.03.99)

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

Name of receiving Office and "PCT International Application":

U.S. Patent and Trademark Office

Box No. I **TITLE OF INVENTION**
Cholesterol Recognition Sequence

Box No. II **APPLICANT**

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

Office of the Dean of Research and Graduate Education
Georgetown University Medical Center
Suite 177, Bldg. D.
4000 Reservoir Road, N. W.
WASHINGTON, DC 20007
UNITED STATES OF AMERICA

[] This person is also inventor.

Telephone no.: (202) 687-1390 Facsimile no.: 202-687-8263

Telecopier no. _____

State (i.e. country) of nationality: US

State (i.e. country) of residence US

THIS PERSON IS APPLICANT FOR THE PURPOSES OF :

[X] all designated states

[] all designated States except the United States of America

[] the United States of America only

[] the States indicated in the Supplemental Box

Box No. III **FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

Papadopoulos, Vassilios
15417 Peach Leaf Drive
North Potomac, Maryland 20878
US

[X] This person is also inventor.

Telephone no.: _____ Facsimile no.: _

Telecopier no. _____

State (i.e. country) of nationality: US

State (i.e. country) of residence US

THIS PERSON IS APPLICANT FOR THE PURPOSES OF :

[] all designated states

[] all designated States except the United States of America

[X] the United States of America only

[] the States indicated in the Supplemental Box

[X] Further applicants and/or (further) inventors are indicated on a continuation sheet.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

**CONTINUATION OF Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER)
INVENTOR(S)**

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)

Li, Hua
1319 Fort Myer Drive, Apt 102
Arlington, Virginia

US

[X] This person is also inventor.

State (i.e. country) of nationality: China

State (i.e. country) of residence : US

THIS PERSON IS APPLICANT FOR THE PURPOSES OF :

- [] all designated states
- [] all designated States except the United States of America
- [X] the United States of America only
- [] the States indicated in the Supplemental Box



Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as ☒ agent
☐ common representative

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country).

Pratt & Associates, Inc.
10821 Hillbrooke Lane
Potomac, Maryland 20854
US

Telephone no. (301)294-9171 Facsimile no. (301)294-7357

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.



Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

Regional Patent

☐ AP ARIPO PATENT: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT.

☐ EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT.

☒ EP European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT.

☐ OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Cote d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member of the OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line)

National Patent (if other kind of protection or treatment desired, specify on dotted line):

- | | |
|---|---|
| <input type="checkbox"/> AL Albania | <input type="checkbox"/> LT Lithuania |
| <input type="checkbox"/> AM Armenia | <input type="checkbox"/> LU Luxembourg |
| <input type="checkbox"/> AT Austria | <input type="checkbox"/> LV Latvia |
| <input checked="" type="checkbox"/> AU Australia | <input type="checkbox"/> MD Republic of Moldova |
| <input type="checkbox"/> AZ Azerbaijan | <input type="checkbox"/> MG Madagascar |
| <input type="checkbox"/> BA Bosnia and Herzegovina | <input type="checkbox"/> MK The former Yugoslav Republic of Macedonia |
| <input type="checkbox"/> BB Barbados | |
| <input type="checkbox"/> BG Bulgaria | <input type="checkbox"/> MN Mongolia |
| <input type="checkbox"/> BR Brazil | <input type="checkbox"/> MW Malawi |
| <input type="checkbox"/> BY Belarus | <input type="checkbox"/> MX Mexico |
| <input checked="" type="checkbox"/> CA Canada | <input type="checkbox"/> NO Norway |
| <input type="checkbox"/> CH and LI Switzerland and Liechtenstein | <input type="checkbox"/> NZ New Zealand |
| <input type="checkbox"/> CN China | <input type="checkbox"/> PL Poland |
| <input type="checkbox"/> CU Cuba | <input type="checkbox"/> PT Portugal |
| <input type="checkbox"/> CZ Czech Republic | <input type="checkbox"/> RO Romania |
| <input type="checkbox"/> DE Germany | <input type="checkbox"/> RU Russian Federation |
| <input type="checkbox"/> DK Denmark | <input type="checkbox"/> SD Sudan |
| <input type="checkbox"/> EE Estonia | <input type="checkbox"/> SE Sweden |
| <input type="checkbox"/> ES Spain | <input type="checkbox"/> SG Singapore |
| <input type="checkbox"/> FI Finland | <input type="checkbox"/> SI Slovenia |
| <input type="checkbox"/> GB United Kingdom | <input type="checkbox"/> SK Slovakia |
| <input type="checkbox"/> GE Georgia | <input type="checkbox"/> SL Sierra Leone |
| <input type="checkbox"/> GH Ghana | <input type="checkbox"/> TJ Tajikistan |
| <input type="checkbox"/> GM Gambia | <input type="checkbox"/> TM Turkmenistan |
| <input type="checkbox"/> GW Guinea-Bissau | <input type="checkbox"/> TR Turkey |
| <input type="checkbox"/> HU Hungary | <input type="checkbox"/> TT Trinidad and Tobago |
| <input type="checkbox"/> ID Indonesia | <input type="checkbox"/> UA Ukraine |
| <input type="checkbox"/> IL Israel | <input type="checkbox"/> UG Uganda |
| <input type="checkbox"/> IS Iceland | <input checked="" type="checkbox"/> US United States of America |
| <input checked="" type="checkbox"/> JP Japan | |
| <input type="checkbox"/> KE Kenya | <input type="checkbox"/> UZ Uzbekistan |
| <input type="checkbox"/> KG Kyrgyzstan | <input type="checkbox"/> VN Viet Nam |
| <input type="checkbox"/> KP Democratic People's Republic of Korea | <input type="checkbox"/> YU Yugoslavia |
| <input type="checkbox"/> KR Republic of Korea | <input type="checkbox"/> ZW Zimbabwe |
| <input type="checkbox"/> KZ Kazakhstan | |
| <input type="checkbox"/> LC Saint Lucia | |

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- | | |
|---|--------------------------------|
| <input type="checkbox"/> LK Sri Lanka | |
| <input type="checkbox"/> LR Liberia | <input type="checkbox"/> |
| <input type="checkbox"/> LS Lesotho | <input type="checkbox"/> |
- In addition to the designations made above, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except the designation(s) of

The applicant declares that those designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit (Confirmation of designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month limit.)



Box No. VI PRIORITY CLAIM

Further priority claims are indicated in the Supplemental Box []
 The priority of the following earlier application(s) is hereby claimed:

Country (in which or for which the application was filed)	Filing Date (day/month/year)	Application No.	Office of Filing (only for regional or international application)
---	---------------------------------	-----------------	---

Item (1) US	12 March 1998 (31.10.97)	60/077,753	
-------------	-----------------------------	------------	--

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required): ☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): (1)

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) (If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):

ISA/ EP

Earlier search Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:

Country (or regional Office): _____ Date (day/month/year): _____ Number: _____

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

- | | |
|------------------------|---------------------------|
| 1. request: 5 sheets | 2. description: 58 sheets |
| 3. claims: 6 sheets | 4. abstract: 1 sheets |
| 5. drawings: 12 sheets | Total: 82 sheets |

This international application is accompanied by the item(s) marked below:

- | | |
|---|--|
| 1. <input type="checkbox"/> separate signed power of attorney | 2. <input type="checkbox"/> copy of general power of attorney |
| 3. <input type="checkbox"/> statement explaining lack of signature | |
| 4. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s): | |
| 5. <input checked="" type="checkbox"/> fee calculation sheet | 6. <input type="checkbox"/> separate indications concerning deposited microorganisms |
| 7. <input type="checkbox"/> nucleotide and/or amino acid sequence listing (diskette) | 8. <input type="checkbox"/> other (specify): |

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Sana A. Pratt
 Sana A. Pratt, Reg. No. 39,441, Agent for the Applicant

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the Applicant ISA/
6. ☐ Transmittal of search copy delayed until search fee is paid



PCT

Fee Calculation Sheet
Annex to the Request

For Receiver's Office Use Only

International application No.

Applicant's or Agent's
file reference 009/0112/SAP

Date Stamp of the Receiving Office

Applicant : Georgetown University Medical Center

CALCULATION OF PRESCRIBED FEES

1. Transmittal fee..... 240.00 (T)

2. Search Fee..... 1,250.00 (S)

International Search to be carried out by EP

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 82 sheets.

first 30 sheets 455.00 (b1)

52 X 10/sheet = 520.00 (b2).

remaining sheets additional amount

Add amounts entered at b1 and b2 and enter total at B..... 975.00 (B)

Designation Fees

The international application contains 5 designations.

5 X 105 = 525.00 (D)

number of designations amount of designation fee
payable (maximum 11)

Add amounts entered at B and D and enter total at I 1,290.00 (I)

4. FEE FOR PRIORITY DOCUMENT 15.00 (P)

5. TOTAL FEES PAYABLE

Add amounts entered at T, S, I and P, and enter total here... 3,005.00

TOTAL

() The designation fees are not paid at this time.

MODE OF PAYMENT

(X) authorization to charge deposit account (see below) () bank draft

() coupons () cheque () cash () postal money order

() revenue stamps () other specify:

DEPOSIT ACCOUNT AUTHORIZATION (this mode of payment may not be available at all receiving Offices)

The RO/ US (X) is hereby authorized to charge the total fees indicated to deposit account NO: 07-1075.

(X) is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to deposit account indicated above.

(X) is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to deposit account indicated above.

Signature

Sana A. Pratt
Sana A. Pratt

U.S. Reg. NO. 39,441

Date

March 12, 1999

